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UNIVERSITÄT ZU SENDAI
AND
MITTEILUNGEN ÜBER ALLGEMEINE PATHOLOGIE
UND PATHOLOGISCHE ANATOMIE

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Vol. 70, No. 3, August 25, 1959

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THE TOHOKU UNIVERSITY

SENDAI, JAPAN

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Y. Satake

YASUTARO SATAKE

Professor Emeritus of the Tohoku University
Member of the Japan Academy

The sad news of the death of Professor Yasutaro Satake shocked and grieved many who knew him. He was born at Tanabe, Prefecture Wakayama, Japan on September 19th, 1884, the first son of Yasuemon Suzuki, a merchant, and of his wife Matsue. He received there his early

education. In 1909 he graduated from the faculty of medicine of the Kyoto Imperial University and soon became assistant in Biochemistry to Prof. T. Araki, who later became President of the University and Privy Councilor. Next year, he became Professor of Physiology at the Kyoto Prefectural College of Medicine and went abroad to make some physiological investigations for about three years under Profs. M. Verworn in Bonn, E. Hering in Leipzig, C. S. Sherrington in Liverpool and Oxford, and I. P. Pavlov in St. Petersburg. In 1915 he was appointed Professor of Physiology at the Tohoku Imperial University, now Tohoku University, and held this post until 1946, when he became President of the University. In 1928 and 1946 he was elected Dean of the Faculty of Medicine. The title of Professor Emeritus was conferred on him one year after his retirement from the University in 1949. In the same year, he was elected Member of the Japan Academy.

Prof. Satake's scientific interests were broad, but he devoted most of his working life to investigating the secretion of adrenaline from the adrenal gland. His interest in this problem was, he told me, aroused by the paper of Drs. J. Negrin and E. Th. Brücke (1914) concerning the effect of Clande Bernard's puncture upon the chromaffinity of the adrenal medulla. At that time, Profs. W. B. Cannon, F. A. Hartman, B. A. Houssay, G. N. Stewart, A. Tournade and others were constantly at work on the secretion of adrenaline. Quantitative estimation of the output rate of adrenaline from the adrenal gland had been achieved only by the " cava pocket " method of Stewart and Rogoff. This method, however, had the disadvantage that anaesthesia and surgical trauma inherent in the method induce hypersecretion of adrenaline. In 1926 Prof. Satake devised an ingenious method for studying the output rate of adrenaline in the unanaesthetized dog. The adrenal vein blood was collected through the lumbar way from the unanaesthetized dog, in which the operation field had been deafferented by section of the posterior spinal roots. The operation was performed without binding the animal to the animal board and without arousing pain. His method made it possible to determine the output rate of adrenaline during rest and under various experimental conditions, and to know the correlation between the secretion of adrenaline, blood pressure, blood sugar, body temperature and other bodily changes. His original observations laid the foundations for our knowledge of the role of adrenaline in the state of mental or physical stress.

The main achievements of his research were published mostly under the names of his associates and pupils, in the *Tohoku Journal of Experimental Medicine*. This recalls to us the ideal of the great physiologist Carl Ludwig whom he adored. His prolific writings were codified and published under the title of *Secretion of Adrenaline and Sympathins* in the Supplement

of the Journal in 1954 and also in the form of a monograph in the next year.

Prof. Satake displayed technical skills in animal operation. He introduced into his country difficult operative techniques of Pavlov for studying the secretion of the digestive glands.

In his condolences on the death of Prof. Satake, Lord Adrian has written that Prof. Satake was for many years a leading figure in Japanese Physiology. And Prof. Houssay has paid a tribute of praise by saying that his original achievements brought glory to his University and his country.

Prof. Satake was a thoughtful lover of his country and his University. His humanism and foresight did not allow him to approve the thoughtless movements of the militarists from the beginning of the second world war. As President of the University he preserved the dignity of the University and raised its prestige in the face of enormous discouragements and difficulties after the war. It owed to his administrative ability that the Faculty of Agriculture was founded in 1947.

Since 1919, he had been Editor of the *Tohoku Journal of Experimental Medicine* and had done much to raise the Journal to a high standard, in collaboration with Prof. T. Kato, now Prof. Emeritus.

It was on his initiative that the Japanese Physiological Society was founded in 1922. The vigorous growth of the Society owed much to his leadership. He edited, with Prof. T. Fujita, now Prof. Emeritus, in 1944 the *Seirigaku-Kōgi*, a textbook of physiology written in Japanese in two volumes. Twelve editions of the first volume appeared in 15 years.

It was Prof. Satake's hobby to give hospitality to his friends and pupils, with the help of his wife. He was familiar with Prof. Cannon through their interests in the problem of the adrenaline secretion. His hospitality given to Prof. Cannon and his wife, who visited Sendai and Prof. Satake's laboratory in 1935, was described elegantly in Cannon's autobiographical writing, entitled *The Way of An Investigator* (1945).

Prof. Satake was fortunate in his marriage. He married, in 1914, Yō Wada who came from Prefecture Nagano, and had two sons, four daughters and fourteen grandchildren.

Prof. Satake was talkative and had not lost his Wakayama dialect, but no one heard his voice rise in anger. His warmheartedness and conscientious fidelity influenced all who came in contact with him. He was sincerely grateful to his teachers, both at home and abroad, until the time of his death.

Prof. Satake died at his home in Sendai on February 14th, 1959 from coronary arteriosclerosis. He was 74 years of age. By his death we have

lost a good teacher and an outstanding physiologist, and we mourn him deeply.

MASAO WADA

The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Studies on the Route and Time of Injection of Donor Cells for the Induction of Acquired Tolerance in the Skin Homotransplantation in Mice

By

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(Received for publication, April 14, 1959)

Based on the concept of acquired tolerance[1], several experiments were conducted on the skin homotransplantation in mice. In the present paper, some new findings concerning the effective route and time through which and at which donor cells were given will be described.

Experimental

Materials and methods

1) Donor and recipient; Adult mice of inbred C57BL/6Ms strain were employed as the donor and the mice of uniform dd-strain were used as the recipient.

2) Skin homotransplantation; 25 to 85 mm^2. of the grafts of free full thickness skin were excised out from the back or both sides of breast of the donor, and each graft was transplanted in the fitted style into the back of the recipient animal by the non-suture method reported in our previous paper[2].

3) Collection of whole blood; A tail of C57BL/6Ms mouse was warmed by hot water (about 40°C) and cut off, the bleeding blood was absorbed into the syringe containing 10 per cent solution of sodium citrate.

4) Preparation of spleen cell suspension; After incision at the upper abdominal region and ligation of the vessels at splenic portal of C57BL/6Ms mice, the spleen was extirpated aseptically under non-anaesthetic condition. Adipose tissue adhered to the surface was removed and then the spleen was compressed carefully in a mortar and suspended in each approximately 0.2 ml. of physiological saline solution.

5) Evaluation of results; Whether the graft which had been homotransplanted was still surviving or not was determined according to the findings obtained by macroscopical examinations and palpation and also to the histological findings.

Results

I. Effects of subcutaneous injections of whole blood or spleen cell suspensions on the homotransplantability

1) Control experiments (Group A):—Seventeen mice of uniform dd-strain were injected subcutaneously on their backs with each 0.06 ml. of physiological saline solution and then the grafts obtained from C57BL/6Ms mice were transplanted 3 or 4 weeks after birth as described above.

As shown in Table I, homografts were sloughed from more than half of the experimental animals in the second week after the transplantation,

TABLE I

Survival Period of the Homografts in Control Experiment (Group A). 17 mice were used

Time after the transplantation (week)	2	5	10
No. of animals with surviving graft	8 (47.1%)	1* (5.8%)	0 (0)

* One graft survived until the 7th week. Average survival period of the homografts was 24.4 days.

and only in one case the graft survived until the seventh week. The average survival period was 24.4 days. Growing of the black hair inherent in the donor could not be observed in any one of this group.

2) Effect of subcutaneous injection of the whole blood of the prospective donor; Fifty-four mice of dd-strain 8 to 24 hours after birth were injected subcutaneously on their backs with each 0.06 ml. of the whole blood obtained from adult mice of C57BL/6Ms strain. Then 3 or 4 weeks after birth these animals were transplanted with the grafts excised from each animal from which whole blood had been collected.

Results are summarized in Table II. Of these 54 animals, homografts survived in 50 cases (92.5 per cent), 36 cases (66.6 per cent), 24 cases (44.4 per cent), 18 cases (33.3 per cent), 16 cases (29.6 per cent), and eight cases (14.8 per cent) 4, 9, 19, 29, 39, and 49 weeks after the transplantation respectively. However, in the 53rd week these grafts sloughed in all cases. Besides, the growing of the black hair was also observed from the second week after the transplantation and in the third week this could be seen in 28 cases (51.8 per cent of total animals and 56.0 per cent of

TABLE II

Survival Period of Homografts in Animals Injected with the Whole Blood of the Prospective Donor. 54 mice were used

Time after the transplantation (week)	5	10	20	30	40	50	60
No. of animals with surviving graft	50 (92.5%)	36 (66.6%)	24 (44.4%)	18 (33.3%)	16 (29.6%)	8* (14.8%)	0

* Of these, 4 grafts survived until the 53rd week.

animals with surviving grafts). After the fourth week, however, the percentage gradually decreased and no case was found in the 40th week.

3) Effect of subcutaneous injection of the spleen cell suspensions of the prospective donor (Group B): In this experiment 39 mice were employed. Each one fifth to one fourth of the total volume of the spleen cell suspension obtained from one individual was injected subcutaneously on the back of recipient animal instead of the whole blood.

Table III represents these results. In most of the animals, grafts were absorbed and sloughed until the 35th week after transplantation and in one case it survived even after the 45th week. Growing of black hair

TABLE III

Survival Period of Homografts in Animals Injected with the Spleen Cell Suspensions of the Prospective Donor (Group B). 39 mice were used

Time after the transplantation (week)	5	10	20	30	40	50
No. of animals with surviving graft	32 (82.0%)	27 (69.2%)	14 (35.8%)	5* (12.8%)	1** (2.5%)	0

* Of these, 4 grafts sloughed gradually until the 35th week.

** One graft survived until the 45th week.

was also observed from the second week in some of the grafts and in the fifth week 30.7 per cent of the total animals of this group (12 cases) and 37.5 per cent of animals with surviving grafts had black hair in the grafts.

II. Effect of time of injection on the homotransplantatability

1) Group C:—Twenty-one mice of dd-strain 16 to 18 days after birth were injected subcutaneously with each one third of the spleen cell suspension obtained from one individual of C57BL/6Ms strain, and on the 30th day after birth they were transplanted with each graft excised from the animal from which the spleen cell suspension was prepared.

As summarised in Table IV, homografts could survive until the fifth week in nine cases (42.8 per cent), and only in one case (4.7 per cent) it survived even until the 10th week. However, in the 14th week these grafts sloughed in all cases.

TABLE IV

Comparison of Survival Period of Homografts Transplanted after the Different Time of Injection of Spleen Celle Suspensions with or without X-Irradiation

Treatment		No. of animals with surviving graft		
		5th week	10th week	20th week
Group C 21 mice	Spleen cell suspension was injected on 16th–18th day.	9 (42.8%)	1* (4.7%)	0
Group D 36 mice	X-irradiated every other day for 3 times from 2 days after birth.	9 (25.0%)	0	
Group E 26 mice	X-irradiated every other day for 3 times from 2 days after birth and spleen cell suspension was injected on 16th day.	23 (88.4%)	16 (61.5%)	7 (26.9%)
Group F 23 mice	X-irradiated every other day for 3 times from 2 weeks after birth and spleen cell suspension was injected on the 3rd week.	14 (60.8%)	8 (34.7%)	2 (18.6%)

* This graft sloughed on the 14th week after the transplantation. In each case homotransplantation was performed on the 30th–35th day after birth.

2) Group D:—Thirty-six mice of dd-strain were used. From the second day after birth animals were irradiated with 100 r of X-ray (150 kv, 40 cm., Cu 0.5 mm., Al p.5 mm.) every other day three times. Then on the 32nd day after birth homotransplantations were performed with the grafts obtained from C57BL/6Ms mice.

In this group grafts, as shown Table IV, survived until the fifth week in nine cases (25.0 per cent), however, any of them could not survive until the 10th week after the transplantation.

3) Group E:—Twenty-six mice of dd-strain were irradiated with 100 r of X-ray (150 kv, 40 cm., Cu 0.5 mm., Al 0.5 mm.) every other day three times from the second day of birth, and then on the 16th day these animals were injected subcutaneously with one third volume of the spleen cell suspensions obtained from each prospective donor. After these treatments, experimental animals were homotransplanted with each graft obtained from C57BL/6Ms mice from which spleen cell suspensions were prepared.

As shown in Table IV, grafts were surviving in 25 cases (88.4 per cent), 16 cases (61.5 per cent), and seven cases (26.9 per cent) 14, 9, and

19 weeks after the transplantation respectively. Moreover, growing of the black hair could be observed in six cases (23.0 per cent of the total animals and 26.0 per cent of animals with surviving graft) in the fifth week.

4) Group F :—From the second week after birth, 23 mice of dd-strain were irradiated every other day three times with 100 r of X-ray (150 kv, 40 cm., Cu 0.5 mm., Al 0.5 mm.), and 2 week after birth spleen cell suspensions were injected suscutaneously as in the cases described above. Then on the 34th day after birth these animals were homotransplanted with the grafts obtained from the C57BL/6Ms mice from which spleen cell suspensions were prepared.

Surviving grafts were observed in 14 cases (60.8 per cent), eight cases (34.7 per cent), and two cases (8.6 per cent) on the fifth, 10th and 20th week after the transplantation respectively (Table IV).

Survival periods of homotransplanted grafts in these groups described above are compared in Fig. 1. Although, the survival period of the grafts

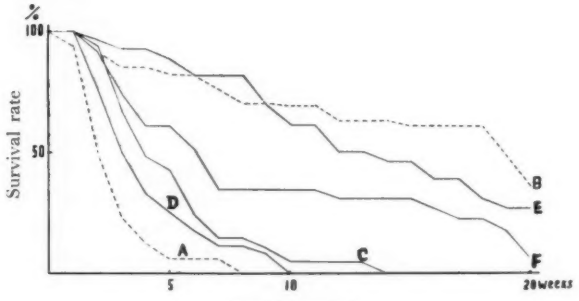

Fig. 1. Comparison of survival period of homografts transplanted after the different time of injection of spleen cell suspension with or without X-irradiation. (see Text)

in Group C (spleen cell suspension was injected 16 to 18 days after birth and homotransplantation was performed on the 30th day) was considerably shorter than that in Group B (spleen cell suspension was injected 8 to 24 hours after birth and homotransplantation was performed in the fourth to fifth week), this was slightly longer than in the case of Group A (physiological saline solution was injected 8 to 24 hours after birth and homotransplantation was performed in the fourth to fifth week). Thus, there was a significant difference of 37.0 per cent at the level of five per cent between the surviving rates of homografts in Group C and Group A. From this result the effect of subcutaneous injection of the cells of homologous origin could be recognized even on the 16th to 18th day after birth.

Besides, in the case of Group E which had been irradiated shortly after birth prior to the injection of the donor cells which was performed on the 16th to 18th day, the survival period was prolonged almost as much as in Group B. Thus, no significant difference was observed between surviving rates of the homografts in Group E and Group F at the level of five per cent.

Next, considerable prolongation of survival period was also observed in Group F which had been irradiated from two weeks after birth and in the third week the spleen cell suspensions were injected. However, in Group D which was irradiated soon after birth but was not injected with the spleen cell suspension prior to the homotransplantation, little significant prolongation was observed as compared to Group A; in the fifth week no significant difference of surviving rate between both groups was observed at the level of five per cent.

Discussion

In the case of skin homotransplantation, it is recognized that the route and time of injection of the donor cells which can induce the tolerance into recipient differ depending upon the kind of experimental animals. Thus, Woodruff and Simpson[3][4] reported that in the case of rats tolerance could be induced also by the subcutaneous injection of donor cells, and that effective period of time was until about the second week after birth. In the case of mice, however, it was reported by Billingham *et al.*[5][6] that such a tolerance could not be induced by the subcutaneous injection of the donor cells, and that effective poeriod of injection was at most seven days after birth.

In the present experiments on the skin homotransplantation with C57BL/6Ms mice as donor and the mice of uniform dd-strain as recipients, we succeeded in the establishment of the transplantation which had been performed in the fourth to fifth week after birth injecting mice subcutaneously either with the whole blood or spleen cell suspensions obtained from the prospective donors 8 to 24 hours after birth. Moreover, the injection of donor cells was slightly effective even on the 16th to 18th day after birth. However, the tolerance induced by these treatments did not maintain more than 53 weeks after the homotransplantation.

Besides, concerning the prolongaton of the acquired tolerance range the effect of cortisone treatment prior to the injection of donor cells was studied in mice by Woodruff and Simpson[4], but no significant effect was observed in their experiments.

In the present experiments, the tolerance range was evidently prolonged by X-irradiation which had been performed shortly after birth. However, it remains obscure at the present time whether or not the effect

of X-irradiation observed here is a phenomenon similar to what resulted from the mechanism of so called radiation chimera[7]. In our experiments, however, donor cells were given two weeks after the X-irradiation and neither early mortality nor delayed mortality was observed in the recipients. From this fact some other mechanism may be considered as the cause of this phenomenon.

Summary

1) Skin homotransplantation was performed in the fourth to fifth week after birth of uniform dd-strain mice which had been injected subcutaneously with the whole blood or spleen cell suspension obtained from the prospective donors (C57BL/6Ms mice) 8 to 24 hours after birth. It was successful in considerably high percentage. Homografts survived for a long period of time and the growing of black hair inhered in the donor was also observed in many cases.

2) Subcutaneous injection of the cells from homologous origin was effective even after 16 to 18 days after birth.

3) If the recipients were X-irradiated shortly after birth, the tolerance range was prolonged evidently against controls without irradiation.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Some Analyses of Slow Potentials of Toad's Retina

By

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(Received for publication, April 15, 1959)

Since the discovery of the electroretinogram by Holmgren in 1865 workers have attempted to clarify the mechanism of its generation. The ERG is a compound response. Granit[1] separated three processes PI, PII and PIII by the effects of narcotics and other chemical agents, and they have been admitted widely to-day. The sources of these components within the retina, however, were not clear, though Granit[1] and others reported some opinions about them. Recent development of the microelectrode technique has made possible the recording of focal responses in every depth of the retina with microillumination. Tomita[2], Svaetichin[3], Brindley[4] and other workers have analyzed retinal responses by this method. The inverted retina preparation devised by Svaetichin is convenient to use for this purpose. Noell[5], on the other hand, has analyzed the ERG from the electrophysiological and histological effects of azides and iodates. Svaetichin[2] and Brindley[4] claimed that the ERG was elicited from the receptor layer and that focal responses made no contribution to the usual ERG. Svaetichin[6] ascribed components of opposite polarity to cones and rods. Noell[5] regarded the receptor layer as the sources of PII and PIII, while Tomita[7][8] claimed that at least a part of the ERG originated in secondary neurons, especially bipolar cells.

In the present investigation some experiments on slow potentials of toad's retina were done with the microtechnique, and some analysis was attempted of the so obtained experimental results.

Experimental

Method

Experiments were made on retinae of toads (*Bufo vulgaris*). Preparations were made in daylight or under rather weak room illumination. Toads were kept in a dark room several hours before ophthalmectomy. After removing the pigment epithelium an inverted retina was mounted

with the receptor side upwards on a piece of black cloth soaked with saline. An Ag-AgCl electrode was set beneath the cloth to be used as an indifferent electrode. A KCl-filled microelectrode was inserted from the surface of the receptor side, but in some experiments (see Fig. 1) a wick electrode was used. The intensity of light was about 4,000 lux and in several experiments microillumination was made with a small light spot 100 × 150 μ in size. When a smaller spot was used in a part of experiments, an image of the filament of a light source placed on the ocular side of a microscope was focused on the retina through the microscope. Thus a spot as small as 20 μ in diameter and 6,000 lux in intensity was obtained. The recording was made by means of a D.C.-amplifier with a cathode-follower input and a cathoderay oscilloscope. Sometimes a C.R.-coupled amplifier with a time-constant of 1.5 seconds and a pen-writer were used.

Results

1. *Contribution of the pigment epithelium to slow potentials*

As the pigment epithelium is usually removed in the inverted retina preparation, the question arises as to whether the response obtained from the inverted retina is unnatural. So the typical slow potential of the inverted retina was compared with that obtained from the eyebulb preparation in which the pigment epithelium remained intact. Fig. 1, A shows a response to even illumination. In this experiment a wick-electrode was placed on the inner surface of the retina, and the positivity of the electrode was directed upwards. An inverted retina preparation was prepared from the same eye after taking record A, and the record shown in Fig. 1, *a* was obtained. For convenience of comparison the negativity of the wick-electrode on the receptor side is directed upwards in this record. In general, both records A and *a* resemble one another. The upward deflection at " on " is the b-wave, which can be preceded by a downward deflection usually called the a-wave. In the usual ERG the b-wave is followed by a slow c-wave. It is to be noted that the record which was obtained from the inverted retina without the pigment epithelium lacks the c-wave. The pair of records B and *b* represent another example. The record B obtained from the eyebulb shows a remarkable c-wave, while it is absent in the record *b*. The difference was investigated on more than ten preparations and there were little departures.

Noell[5] claimed that c-wave was due to the pigment epithelium, and the results obtained above support his opinion.

The amplitude of response, especially of the off-response (d-wave) was often larger in the inverted retina. Some inverted retinae showed a deep a-wave and a small b-wave, but this seemed to be caused from some

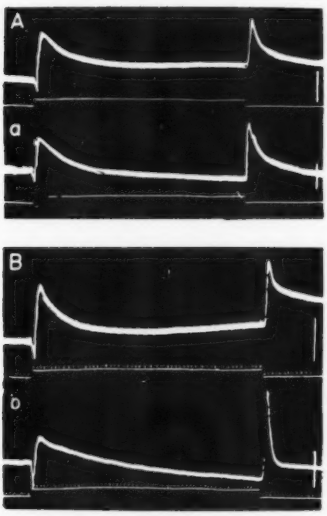

Fig. 1. A and B show responses recorded from opened excised eye. Positivity of wick-electrode placed on inner surface of retina is directed upwards. *a* and *b* show responses recorded from inverted retina. Negativity of wick-electrode placed on outer surface of retina is directed upwards. Vertical bar indicates 300 μV, and stimulus duration is about 6 seconds.

damage in removing the pigment epithelium.

2. *Types of response*

In the following experiments exclusively inverted retinae were used, and the polarity of response was referred to the microelectrode placed on the receptor side of the preparation. As has been shown in Fig. 1, the a- and b-waves of ERG correspond with positivity and negativity of the microelectrode on the receptor side respectively, but such notations a, b, c and d will not be used for records obtained from inverted retinae, because the properties of such records are different in many respects from those of the usual ERG, for example, the ERG is diffuse in character, while the response recorded by a microelectrode to microillumination is focal in character. The positivity is directed upwards in all the following records.

Under some conditions responses lacking the negative component were obtained (see Fig. 2). When the pigment epithelium was removed carelessly so that the surface of the receptor side was possibly damaged, this type of response was obtained to even illumination. This type will be called positive type. Sometimes a part of retina showed this type and the other parts showed normal or negative type. In winter and especially in early

spring inverted retinae of toads which passed hibernation were so apt to show the positive type that it was difficult to obtain the normal type in spite of careful treatment. The possibility of obtaining the normal type was not increased even if toads were kept at room temperature of 20°C about ten hours before experiments, or preparations were warmed to 25°C. Svaetichin[3] reported that the b-wave of the frog's retina was not absent under such low temperature as 1°C. Several toads were fed on toad's liver or injected 0.5 c.c. of liver oil into the peritoneal cavity 48 hours before experiment, but none of their retinae showed a complete negative type. To pour saline on a preparation showing responses of the positive type was found sometimes useful to change its response type. Bernhard[9] and Deane *et al.*[10] reported a similar type of ERG of turtles which were in hibernation. But responses recorded from opened excised eyes of toads did not show the positive type so often as responses of inverted retinae. A piece of retina minced about 1 mm² in area showed responses of the positive type as investigated by Ottoson and Svaetichin[11].

In some preparations showing responses of the positive type a negative deflection appeared at cessation of light as in Fig. 2, B. When the duration of the stimulus was short, no "off"-response appeared in normal type but did in positive type.

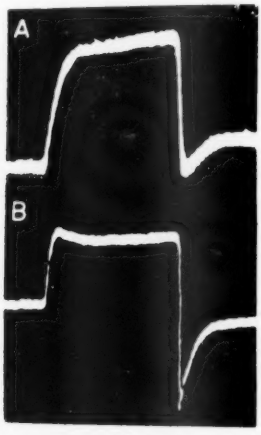

Fig. 2. Some response types of inverted retina. Positivity of electrode placed on outer surface of retina is directed upward.

Next, responses to microillumination will be mentioned. As has been shown in Fig. 1, the response recorded from the surface of the inverted retina to even illumination was very similar to the usual ERG and this relation changed little when a spot of stimulus light as small as 20 μ in

diameter was used (see Fig. 4 or Fig. 7). The type of response to micro-illumination usually suffered from a considerable change by touching the surface of the inverted retina with a piece of dry filter paper; Responses of the negative type before the treatment turned into responses of the positive type. Tomita found a similar effect (personal communication), and Pilz *et al.*[12] reported also a similar change which they considered to be due to damage. From what has been observed above it is likely that the positive type appears when the receptor surface is damaged in some way or other.

3. *Type of response and adaptation state*

Sometimes a negative going deflection was superimposed on the basic positive response (see Fig. 3A). In this experiment the stimulus light

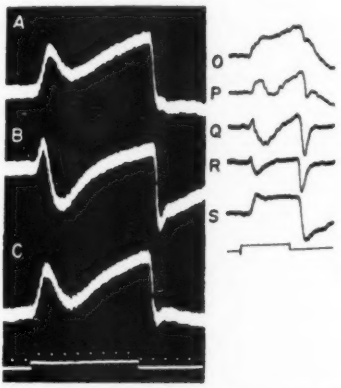

Fig. 3. Effect of light adaptation on responses of inverted retina.
A, C: Responses under dark adaptation.
B: Response under background illumination of 20 lux.
O: Dark; P: 2 lux; Q: 5 lux; R: 20 lux; S: 5 lux. Positivity upwards. Time mark: 10 cps.

spot was 1×1 mm. in size. The negative response became larger when the preparation was light-adapted to background illumination of 20 lux (see Fig. 3B). When the adapting light was turned off, a response of the original shape was obtained again (see Fig. 3C). Such a difference due to background illumination was distinct in preparations made from toads which had been light-adapted for several hours. In spring one preparation made from a toad kept long in sunshine showed response of the positive type in dark adaptation, but those of negative type in light adaptation. The records in Fig. 3, O–S show this relation. The stimulus light spot was 20 μ in diameter and 6,000 lux in intensity in all the records and the intensity of background illumination was 0, 2, 5 and 20 lux for O, P, Q

and R respectively, and it was reduced to 5 lux in S after R had been recorded. But in S the negative deflection was only slight compared with Q, although background illumination for Q and S was identical.

4. *Properties of focal responses*

It seemed that the type of response was characteristic of the locus from which the potential was recorded. A microelectrode placed on a locus damaged by a touch with filter paper picked up responses of positive type, while a microelectrode placed on an intact part around the damaged part showed normal negative responses. The difference in type of the responses at the damaged and intact parts did not disappear when the preparation was subjected to even illumination. This finding suggests that a microelectrode can pick up focal responses. The question arose to what extent a focal response at a locus spread into the surrounding area. To answer this question, the following experiments were carried out. A microillumination of 20 μ in diameter was used. The intensity was 6,000 lux, and the background illumination 15 lux. First a locus on which the microelectrode was placed was illuminated and a normal response consisting of remarkable negative "on" and "off" deflections was obtained (see record labeled 0 mm. in Fig. 4). When the microillumination was dis-

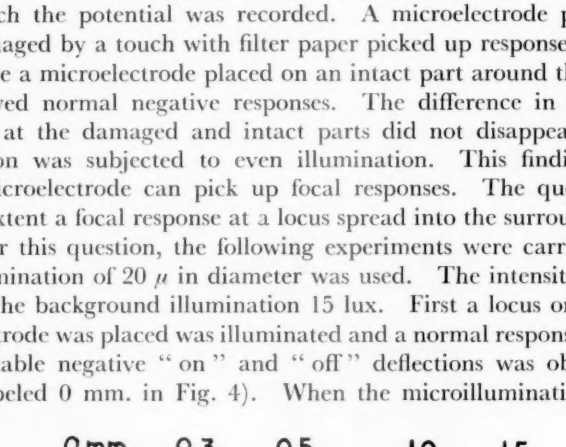

Fig. 4. A: Dependence of response on distance between recording electrode and microillumination. Numerals above records show distances. B: Similar dependence when mechanical cut was made at 0.3 mm. Time scale indicates 100 msec.,

placed to points 0.3, 0.5, 1.0 and 1.5 mm. distant from point 0, the records labeled 0.3, 0.5, 1.0 and 1.5 in series A of Fig. 4 were obtained respectively. It is apparent from these records that the magnitude of response decreases with increasing distance from the illuminated locus, and that no response is perceived at a distance of 1.5 mm.

Next, a shallow cut was made with a blade of razor at a point 0.3 mm. from the electrode. When illumination was again centered on the electrode, a response very similar to that taken before was obtained, although

the amplitude was a little smaller (see series B of Fig. 4). When the site of cut was illuminated, a response of positive type was recorded, and when the light spot illuminated a point on the other side of the cut at a distance of 0.5 mm. from the electrode, a small potential of similar shape was obtained, but none could be recorded when a point 1 mm. away from the electrode was illuminated.

It is possible that responses recorded outside the illuminated spot are due to stray light, but the experiment of cut in series B of Fig. 4 will be difficult to interpret in terms of stray light. The point 0 at which the electrode was placed was not damaged by the cut, because a normal negative response was obtained to illumination of this point. However, by the same electrode at 0 a response of positive type was recorded when the damaged part at 0.3 mm. was illuminated. Therefore, the potential can not be a response to stray light at the site of the electrode. It must be due to either electrotonic spread from the damaged part or physiological spread.

Pilz *et al.*[12] found responses of positive type at a damaged part which approached the normal type when a neighboring intact part was illuminated. This may be due to electrotonic spread. In similar experiments made on the anterior side of the isolated retina responses were reduced in maganitude by a cut, but normal in shape. It was, however, uncertain because of the vitreous body, if the important part of the retina was damaged by the cut.

The effect of stray light was eliminated in the following experiments shown in Fig. 5. A represents a control taken under dark adaptation. The lower tracing represents a response at the illuminated site and the upper one a response at a distance of 1 mm. from the illuminated locus. A background illumination of 40 lux reduced both responses (B). Record C was obtained when the locus from which the upper tracing was obtained was continuously illuminated by a light spot of the same size and intensity as the stimulus light spot. Under such strong continuous illumination the effect of stray light is obviously negligible so that the response represented by the upper tracing can not be due to stray light. For record D a background illumination of 40 lux was added to the local constant illumination used in C.

These experiments show that background illumination is effective in reducing the magnitude of responses, while a small local adaptation is almost ineffective. If response recorded at a distance of 1 mm. from the stimulated site were due to electrotonic spread, it could be expected that the upper and lower tracings are identical in form. As a matter of fact, however, a considerable difference can be seen between the latencies of both tracings.

5. *Lissajous' figure of responses at different loci*

Two microelectrodes were arranged on the surface of the inverted retina at a distance of 1 mm. apart to record responses simultaneously. The upper record in Fig. 6A represents responses to flickering light at the illuminated locus and the lower record those obtained from the unilluminated locus. The stimulus was $100 \times 150\ \mu$ in size and its intensity 4,000 lux. The frequency of flicker was 18 cps, and light to dark ratio was 1:1.

Fig. 5

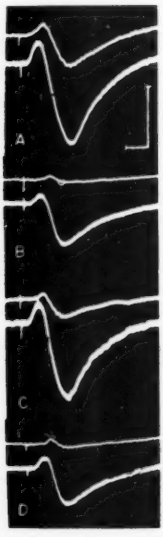

Fig. 6

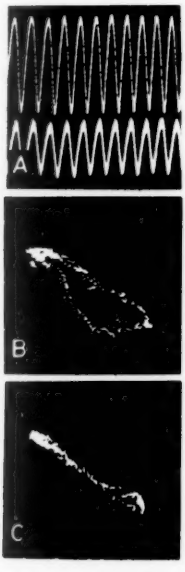

Fig. 5. Simultaneous records from two electrodes arranged 1 mm. apart. Each lower tracing represents response to a light spot $100 \times 150\ \mu$ in size at the center of illumination. Stimulus intensity was 4,000 lux. Each upper tracing represents response recorded by other electrode. A: Recorded without background illumination. B: Recorded under background illumination of 40 lux. C: Recorded withbut background illumination, but the tip of remote electrode was constantly illuminated with light spot of the same size and intensity as stimulus spot. D: Recorded under background illumination of 40 lux, otherwise under same experimental conditions as C. The beginnings of stimuli are indicated by shock-artifact. Vertical bar indicates 500 μV and horizontal bar 100 msec.

Fig. 6. A: Upper recod indicates response at illuminated locus to intermittent light of 18 cps, and lower one response at a distance of 1 mm. B: Lissajous' figure recorded by upper and lower responses in A. C: Lissajous' figure recorded by two responses at points 1 mm. apart subjected to common illumination.

A Lissajous' figure was recorded with the two kinds of response mentioned above (see B in Fig. 6). The figure indicates that there was a certain phase difference between the response at the illuminated locus and that obtained from the unilluminated neighboring part. For control a Lissajous' figure was recorded by responses to intermittent illumination common to both electrodes. This finding is in line with the experiment shown in Fig. 5.

6. *Dependence of slow potentials on depths*

The potential of positive type can be seen after the superficial layer of the inverted retina has been damaged. If it is assumed that the receptor layer is damaged by this treatment, the potential of this type must originate in some layer other than the receptor layer.

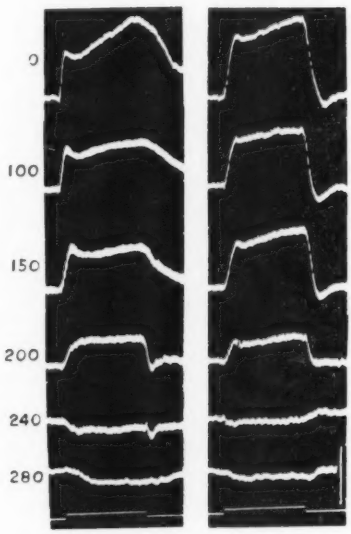

Fig. 7. Responses of positive type at various depths of retina. Left column concerns responses to even illumination and right one responses to micro-illumination. Numerals indicate apparent depths in μ from receptor side.

Tomita *et al.*[7] inserted a microelectrode into the retina and found that PIII component which was isolated by potassium reversed its polarity when the electrode reached a depth of a layer external to the bipolar cell layer. He regarded this finding as evidence that the major part of PIII was originated in the bipolar cell layer.

The records in Fig. 7 which were obtained at room temperature of 20°C show responses recorded by a microelectrode inserted into the retina

from the surface of the receptor layer where damage was made with filter paper. The left column represents responses to even illumination and the right one those to microillumination and numerals show depths in μ from the surface. The "off" swing was slower in records taken from layers near the surface, but such was not a constantly observable finding. When the electrode was inserted to a retinal layer, possibly the inner plexiform layer, at a depth of 200–240 μ the response was found to reverse its polarity. The location of change in polarity did not show a difference greater than ± 30 μ in a number of such experiments. The reverse in polarity suggests that the origin of the response of positive type may be in the inner nuclear layer.

Fig. 8 shows normal responses recorded in similar way from a not

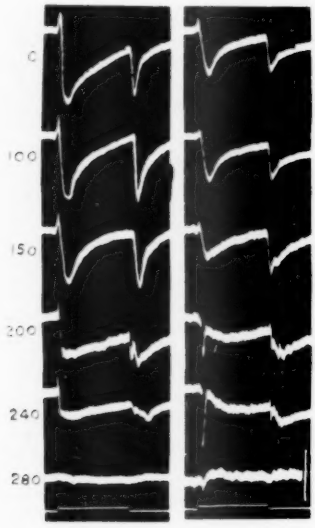

Fig. 8. Responses of negative type at various depths of retina. Left and right columns concern responses to even and focal illuminations respectively.

damaged retina. The response type was negative both to even illumination and microillumination upto the depth of 150 μ, but a change occurred at depths of 200–240 μ especially in responses to microillumination, the slow negative response to microillumination was preceded by a spike-like nagative deflection, while such a negative sharp deflection was absent in the response to even illumination. Judging from the latency this negative sharp deflection did not seem to be a reversed on-positive response, and never appeared in the response of positive type. When the light spot was moved 0.5 mm. away from the electrode this deflection was lost sight

of so that the response became similar to that to even illumination.

When the electrode was advanced into a retina showing responses of positive type to microillumination (see A in Fig. 9) a change in polarity occurred at a depth of about 250 μ from the surface of the receptor side and spikes appeared superimposed on the slow negative potential. (see in Fig. 9).

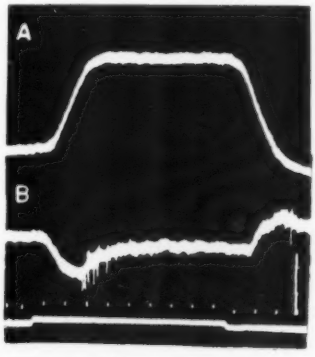

Fig. 9. A: Response of positive type on receptor surface. B: Response accompanied by spikes at a depth of 250 μ from receptor side.

Discussion

It has been shown above that the responses obtained by a microelectrode to microillumination are focal in character and are different from those of the usual ERG. But local responses to microillumination are always very similar in shape to the ERG when they are recorded from the surface of the inverted retina. So on the receptor surface focal response and ERG may be picked up from the common origin. The additivity in area-effect on ERG found by Brindley[13] is one of evidences for this conjecture. But the responses recorded in the depth of more than 100 μ are different from those recorded from the receptor surface. They are intraretinal focal responses.

It is possible that the response of positive type is identical with PIII. When 1% solution of KCl is dropped on the inverted retina or when the preparation is laid in an atmosphere containing 10% CO_2 a change in response type occurs from normal negative type to positive one. This change is the same as in isolation of PIII from the ERG. The origin of the response of this type seems to be in the intermediate layers such as the inner plexiform layer and inner nuclear layer. Tomita[7] assumed that its origin is in the bipolar cell layer. A response of positive type was obtained even when a shallow cut was made between the stimulus spot and the recording

electrode on the receptor surface. This finding suggests that a deeper layer must be responsible for PIII.

The phase difference indicated in the Lissajous' figure suggests a possibility of propagation, although farther evidence is needed for this notion.

It has been said that the b-wave and PIII are due to rods and cones respectively. Deane *et al.*[10] reported, however, that the cone retina of the turtle had a distinct b-wave and that it was larger in amplitude to red stimulus light than to green one. From their experiment on flicker Dodt & Enroth[14] considered that the a-wave expressed rod activity while the b-potential following high flicker frequency was due to cone activity. In the experiment mentioned above the negative type which was assumed to be identical with the b-wave was obtained more easily in light adaptation than in dark adaptation. This fact seems to suggest that the negative and positive types are associated with cone and rod activity respectively.

It was shown above that even when the response recorded from the receptor surface lacked the negative component, spikes appeared in the record obtained from a deep layer, possibly the ganglion cell layer. So the spikes in this layer seem to be more closely related to the slow potentials of positive type obtainable from the receptor side than to those of negative type. Tomita *et al.*[15] and Gouras[2] reported that ganglion cell activity was associated with surface negative waves. They used opened excised eyes, so that the surface-negative wave may be equivalent to the response of positive type on the inverted retina.

It was assumed by Granit[1] that the off-response of ERG was produced by the return of PIII to the baseline. The off-response could be seen in the response of positive type. If this type of potential is identical with PIII, it is apparent that the cause assumed by Granit is not the only one available for interpretation of the off-response. On the other hand, Tomita[7] said that one part of the off-response had its origin in a layer common to the b-wave. In the present experiment sometimes a part of the inverted retina showed responses of positive type while the other parts showed those of negative type as mentioned above. In such preparations the off-responses obtained from these parts different in character showed little regional difference.

Summary

A KCl-filled microelectrode was inserted into toad's inverted retina to record slow potentials to microillumination or to even illumination.

1. Responses obtained from an inverted retina was compared with those recorded from an opened eyebulb, and from this experiment it was suggested that the pigment epithelium contributes to the c-wave of ERG.

2. Responses were divided into two types; positive and negative.

It was possible to obtain the positive type by giving a slight damage to the receptor surface. The negative type was obtainable more easily in light adaptation than dark adaptation.

3. Properties of focal responses to microillumination were examined. In experiments in which a mechanical cut or twin stimulus spots were used it was confirmed that the spread of potential over 1 mm. from the illuminated focus was not due to stray light. The latency of the response obtained at the illuminated spot was found shorter than that of the response obtained from a point of the surrounding area, as evidenced by comparison of the two records or by Lissajous' figure.

4. Slow potentials of positive and negative types were recorded from different layers of the retina and compared with similar experiments by previous workers.

5. It was discussed on the origin of each component of ERG.

I am greatly indebted to Prof. K. Motokawa for his kind guidance and for many valuable discussions.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Evidence for the Action of Incomplete Antibodies in Experimental Immunohemolytic Anemia

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(Received for publication, April 23, 1959)

Since Dameshek and Schwartz[1] produced a hemolytic anemia with microspherocytosis, increased osmotic fragility of erythrocytes and reticulocytosis in guinea pigs by injecting anti-erythrocyte serum in 1938, and attributed these effects to the action of hemolysins, it has been generally accepted that complete antibodies such as hemolysins[2], hemagglutinins[3][4][5][6] and hemopsonins[2][7][8] play significant roles in the mechanism of the immunohemolytic anemia.

Although it is well known that incomplete antibodies play some etiologic roles in acquired hemolytic anemia in man[9], this has never been demonstrated experimentally in immunohemolytic anemias. We have studied the contribution of incomplete antibodies in an immunohemolytic anemia.

Experimental

Method

I. Production of anti-erythrocyte serum

Seven to ten injections, spaced three days apart, of three ml of 40% rabbit erythrocytes suspension in saline were given to dogs. When the hemolysins and hemagglutinins titers reached a maximum, the blood was collected and antiserum was prepared.

II. Production of Coombs' test serum[10][11]

Injections of above-mentioned anti-serum were given to ducks at three days interval for a total of seven injections. One week after the last injection, the precipitin titer for the anti-erythrocyte serum was measured. If the titer was sufficiently high, the blood was collected from the duck. The duck serum was inactivated, mixed with ertyrhocytes from several rabbits and held at 37°C for two hours to adsorb the heterohemagglutinins to the rabbit erythrocyte. Rabbit erythrocytes were then separated by centrifugation and discarded. The supernatant anti-anti-erythrocyte

serum duck serum was used for Coombs' test serum.

III. Hematologic examination.

After the injection of anti-erythrocyte serum into rabbits, the blood of these animals was studied at intervals to determine the red blood cell counts, hematocrits, hemoglobins, reticulocyte counts, osmotic fragility of erythrocytes and Price-Jones' curve. The myelogram was also examined at interyals.

IV. Serologic Tests

Hemolysins and hemagglutinins in the blood of rabbits receiving injections of anti-serum were examined at intervals. The incomplete antibodies attached to erythrocytes from rabbits were determined using the above-mentioned Coombs' test serum. Indirect Coombs' test was also performed.

V. Erythrocytes survival test was performed using the Cr^{51} method.

VI. The histopathologic findings of liver and spleen are examined.

Results

I. The experimental immunohemolytic anemia in rabbits due to the injection of anti-erythrocyte serum.

Ten ml of anti-serum containing hemolysins of a titer of 560 and hemagglutinins of 2640, injected intravenously into rabbits.

1. The osmotic fragility of red blood cells, as shown in Table I, increased markedly within few hours after the injection, and remained low for about two weeks.

TABLE I

The Change of Osmotic Fragility of Red Blood Cells after the Intravenous Injection of Anti-erythrocytes Serum

Rabbits No.	Time before	3–6 hours after	1 day after	5 days after	7 days after	9 days after
No. 1	0.54–0.40	0.58–0.46	0.58–0.46	0.58–0.44	0.56–0.44	0.54–0.42
No. 1	0.54–0.40	0.58–0.46	0.58–0.46	0.58–0.44	0.56–0.44	0.54–0.42
No. 2	0.52–0.30	0.60–0.46	0.60–0.46	0.58–0.44	0.56–0.44	0.56–0.42
No. 3	0.54–0.42	0.60–0.50	0.60–0.50	0.56–0.44	0.56–0.44	0.56–0.44
No. 4	0.56–0.42	0.60–0.46	0.60–0.44	0.58–0.44	0.56–0.44	0.56–0.42
No. 5	0.54–0.38	0.60–0.50	0.60–0.50	0.60–0.52	0.56–0.44	0.56–0.38

2. A marked reduction in red blood cells, hemoglobin content and hematocrit value was noticed in all rabbits within six days after injection. There were no change in reticulocyte count within six hours after the injection. However, the reticulocytes increased markedly 24 hours after

injection and persisted about two weeks.

3. One day after the injection of anti-serum, the Price-Jones' curve had moved markedly to the left, and it had two peaks. Although the peak of the Price-Jones' curve returned to the right two to four days later, a large number of microcytes was still found.

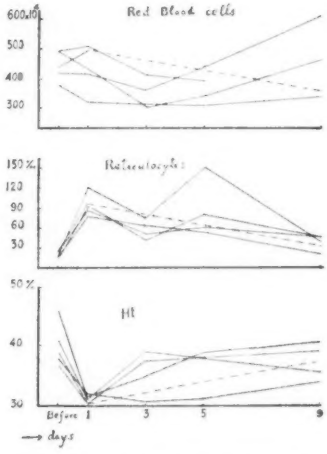

Fig. 1. Changes of red blood cells, reticulocytes and Ht after injection of anti-erythrocytes serum.

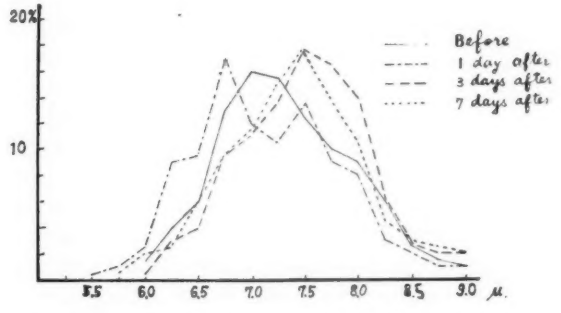

Fig. 2. Changes of price-jones curve after the injection of anti-serum.

4. Morphologically microcytes and spherocytes increased 24 hours after the injection. Although there was an increase of large red blood cells identified as reticulocytes, three to nine days after the injection, many spherocytic microcytes were also seen.

6. Myelogram:

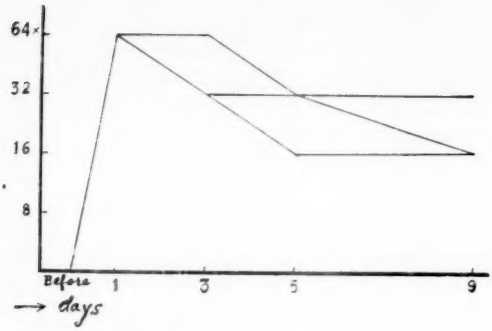

Fig. 3. Coombs' test titer.

There were no changes in the nucleated cell count. The myelotic cells were not altered by anti-serum injection. However, the erythroblasts were greatly affected. There was a marked proliferation of basophilic and polychromatic erythroblasts. Mitoses of erythroblasts were also often observed (Table II).

In short, we found that the intravenous injection of anti-serum produced no effect of myelotic cells but did produce an increased erythropoiesis.

7. Serologic tests:

Hemolysins, hemagglutinins and incomplete antibodies were never found in the blood of rabbits receiving the injection of anti-erythrocyte serum. The indirect Coombs' test was negative in these rabbits. However, antibodies attached to erythrocytes were always detected for about two weeks by the direct Coombs' test.

II. Acute immunohemolytic anemia in rabbits due to the injection of heat-treated anti-serum containing no complete antibodies.

1. Production of anti-serum containing only incomplete antibodies

We heated the anti-erythrocyte serum containing complete antibodies such as hemolysin and hemagglutinin as well as incomplete antibodies at 65°C for 60 minutes. There was no activity of hemolysins and hemagglutinins after this heating. However, incomplete antibodies were still demonstrated by indirect Coombs' test.

Ten to fifteen ml. of this heat-treated anti-serum containing only incomplete antibodies were injected intravenously into rabbits. The above-mentioned examinations were then made.

2. The osmotic fragility of red cells increased 24 hours after the injection, and remained elevated for about six days (Table III).

3. The red blood cells counts reduced slightly within six days after the injection. The hematocrit value decreased remarkably 24 hours after

TABLE II

The Change of Myelogram after the Intravenous Injection of Anti-erythrocytes Serum

		Before	1 day after	3 days after	6 days after
Counts of nucleated cells		107,400	37,200	105,000	82,800
Proerbl		0.4	0.2	0.2	1.4
	Basophilic	2.6	2.0	2.0	4.6
Macrbl	Polychromatic	3.8	1.0	0	0
	Orthochromatic	0	0	0	0
	Basophilic	2.8	5.6	8.8	6.6
Normbl	Polychromatic	42.0	62.8	69.0	68.8
	Orthochromatic	4.8	2.4	4.2	2.4
Total of Erbl.		56.4	74.0	84.2	83.8
Mitosis		1/1000	10/1000	12/1000	10/1000
Mbl		1.6	0.2	0.2	0.4
	Eosin	0	0	0	0
Pro M	Pseudoeosin	1.6	0.2	0.4	0.4
	Eosin	0	0	0	0
M	Pseudoeosin	4.8	1.4	1.2	1.0
	Eosin	0	0	0	0
Meta M	Pseudoeosin	7.0	1.6	0.8	0.8
	Eosin	0	0	0	0
Stabb. L	Pseudoeosin	6.8	2.4	1.6	2.4
	Eosin	0	0	0	0
Seg. L.	Pseudoeosin	12.2	6.6	3.8	4.0
Baso.		0	0	0	0
Total of Myelocytic cells		34.0	12.4	8.0	9.0
Lymph	Large	0.8	0.2	0.2	0.4
	Small	8.2	13.6	7.4	6.4
Total of Lymphocyte		9.0	13.8	7.6	6.8
Monocyts		0.2	0.2	0	0
Plasma cells		0.4	0	0.2	0.4
Reticulum cells		0	0	0	0
Megakaryocyte		1/1000	1/100	1/1000	1/1000
E/M		1.66/1	5.96/1	10.52/1	9.31/1

Notes: Proerbl: Proerythroblast, Macrbl: Macroblast, Normbl: Normoblast Mbl: Myeloblast, Pro M.: Promyelocyte, M: Myelocyte, Meta M: Meta myelocyte.

the injection and it remained reduced for about five days. The reticulocytes increased and remained increased for about one week (Fig. 4).

4. Price-Jones' curve.

Although the peak of the Price-Jones' curve did not change after injection, the per cent of microcytes increased greatly as shown in Fig. 5. This microcytosis continued for about five days. Spherocytosis was also found.

5. Changes of the myelogram due to the injection of the heat-treated

TABLE III

The Increase of the Osmotic Fragility of Red Blood Cells after the Injection of the Heat-Treated Antierythrocyte dog serum

Rabbit No. \ Period	Before	1 day after	3 days after	6 days after
No. 11	0.60–0.30	0.66–0.40	0.66–0.42	0.64–0.42
No. 12	0.62–0.32	0.68–0.42	0.68–0.42	0.64–0.38
No. 13	0.62–0.34	0.66–0.40	0.66–0.40	0.64–0.38
No. 14	0.62–0.30	0.66–0.42	0.66–0.42	
No. 15	0.52–0.32	0.66–0.40	0.66–0.42	0.54–0.40
No. 16	0.60–0.32	0.62–0.42	0.62–0.42	
No. 17	0.60–0.34	0.62–0.42	0.62–0.42	
No. 18	0.60–0.34	0.64–0.46	0.64–0.46	0.55–0.34

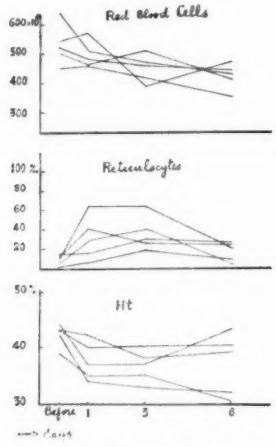

Fig. 4. Changes of red blood cells, reticulocytes and Ht after the injection of heat-treated anti-serum.

antiserum are illustrated in Table IV. The myeloic cells showed no sings of being affected by the injection, but erythropoiesis was found to have increased.

6. Hemolysins, hemagglutinins and incomplete antibodies were never found in the serum of rabbits twenty four hours after receiving the heat-treated anti-serum injection. However, the direct Coombs' test showed a positive reaction in these animals 24 hours after the injection and which persisted for about six days (Fig. 6).

7. The effects of heat-treated anti-serum injections on the life span of erythrocytes.

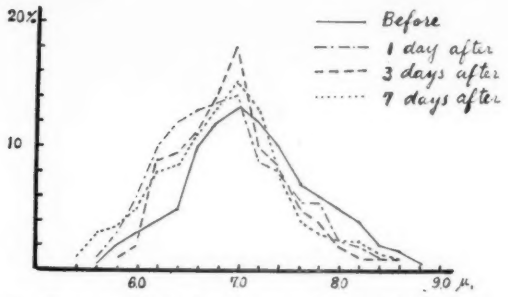

Fig. 5. Changes of Price-Jones' curve after the injection of heat-treated anti-serum.

TABLE IV

The Change of Myelogram after the Intravenous Injection of Heat-treated Anti-erythrocyte Serum

		Before	3 days after	6 days after
Counts of nucleated cells		6,4500	25,2500	4,5500
Mbl		0.4	0.4	2.4
Pro Myel		0.4	1.2	2.0
	Myel	2.8	2.4	3.6
	Meta	3.6	2.8	2.8
Pseudoeosin.	Staffy	4.2	4.8	6.4
	Seg	18.8	15.6	16.4
Eosin.		3.2	1.2	3.4
Baso.		1.2	0.4	0.4
Mono.		0.8	1.6	0.4
Lymphocytes		24.0	25.2	17.6
Plasma cells		2.0	1.6	
Reticulum cells		2.8	2.0	0.4
Proeryth.			0.8	0.8
	Basophile	0	0	8.0
			40.0	45.0
Eryth.	Polychromatic	20.8	19.6	26.2
	Orthochromatic	14.0	19.6	10.8
Mitosis	Eryth.	1.0/1000	6.0/1000	4.0/1000
	Gran	0	0	1.0

Notes: Mbl: Myeloblast, Pro Myel: Promyelocyte, Myelo: Myelocyte, Meta: Meta Myelocyte, Proeryth: Proerythrocyte, Eryth: Erythrocyte.

The heat-treated anti-serum was injected into rabbits and twenty four hours later the survival time of erythrocytes of these rabbits was examined by the Cr^{51} tagged erythrocytes method.[12)13)] The time taken for disappearance of 50 per cent of the Cr^{51} tagged erythrocytes ($T\frac{1}{2}$) was

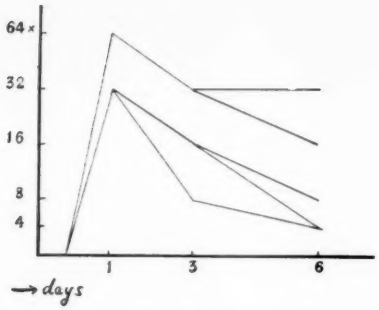

Fig. 6. Coombs test titer after the injection of heat-treated anti-serum.

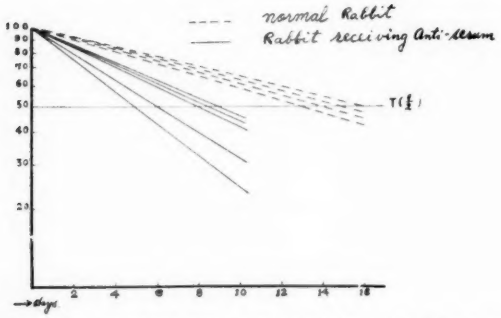

Fig. 7. Life span of red blood cells of rabbits which were given the intravenous injection of heat-treated anti-serum.

13 to 15 days in normal rabbits. The half-time in the rabbits receiving heat-treated anti-serum was five to ten days (Fig. 7).

8. Histologic findings in liver and spleen.

Twenty four hours after the heat-treated anti-serum injection, the spleen showed a marked stasis in the pulp with erythrophagocytosis. Splenic hemosiderosis was also marked. Although the liver showed a marked hemosiderosis 24 hours after the injection, this receded within one week. However, the hemosiderosis in the spleen was still marked seven days after injection.

III. The role of spleen in the elimination of sensitized red cells (Fig. 8)

Five rabbits were splenectomized. Then 10 ml. of heat-treated anti-serum were injected into these rabbits. Slightly increased osmotic fragility, mild microspherocytosis and mild reticulocytosis were observed.

Determination of the life span of erythrocytes by means of Cr^{51} method demonstrated that the life span did not shorten in these rabbits.

IV. Electronmicroscopic examination of the red blood cells membranes

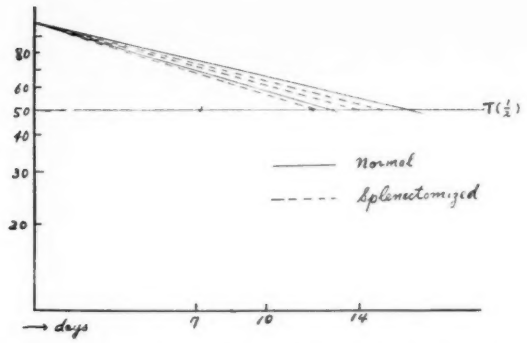

Fig. 8. Life span of red blood cells of splenectomized rabbits receiving heat-treated anti-serum.

which were sensitized by incomplete antibodies.

Suspensions of normal rabbit erythrocytes mixed with the heat-treated anti-serum were incubated for one hour at 37°C. The erythrocytes were washed five times with a physiological saline and then hemolyzed in distilled water. The cell membranes of hemolyzed erythrocytes were examined by an electron microscope. A similar procedure was done for the erythrocytes of rabbits which had received the heat-treated anti-serum injection.

The normal erythrocyte membrane is a thin and finely granular structure, without holes or folds. However, the membrane of the erythrocytes mixed with the heat-treated anti-serum or taken from rabbits receiving the heat-treated anti-serum injection, showed thicknings of the membrane and formation or irregular folds and holes.

Discussion

Since the first experiment was performed to produce hemolytic anemia by heterohemolysin, many authors have recognized possibility that experimental hemolytic anemia may be produced in animals by the injection of anti-erythrocyte serum. However, the hematological findings are incomplete in several respects. For instance, the mechanisms responsible for the appearance of microcytes and for the increase in osmotic fragility of erythrocytes have not been elucidated. Dameshek and Schwartz believed that hemolysins caused the microperocytosis and the increased osmotic fragility.

We gave rabbits intravenous injecion of ten ml. anti-erythrocyte serum. We then checked for changes in hemogram, myelogram, serologic tests and histology of liver and spleen.

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A marked increase in osmotic fragility of red blood cells occurred within three to six hours after the injection. This increased osmotic fragility continued for about two weeks. A marked reduction in erythrocyte count, hemoglobin content, and hematocrit value occurred during the first five days after the injection. The peak of the Price-Jones' curve shifted to the left. Although it returned to the right two for days later, a large number of microcytes was still found. The reticulocytes showed no changes for the first few hours, but then showed anremarkable increase by the end of the first day. The bone marrow showed an increased erythropoiesis, with the number of mitotic figure increasing.

In our experiments, neither hemolysins nor hemagglutinins could ever be demonstrated in the blood of rabbits which received the injection of anti-erythrocyte serum. We could not observe hemoglobinemia and hemoglobinuria in our experiment.

According to past reports on this subject, the hemolytic reactions to hemolysins injection vary depending on the quantity of the hemolysins injected. It is generally believed that while the injection of the large dose of hemolysins causes a fulminating hemolytic anemia with hemoglobinemia and hemoglobinuria due to intravascular hemolysis, that of medium doses produce an acute hemolytic anemia, with spherocytosis, increased osmotic fragility and reticulocytosis. Small doses bring chronic hemolytic anemia in which erythropoiesis of the bone marrow is so remarkable that the hemolytic symptomes may pass unnoticed. There was no evidence of severe intravascular hemolysis in our experiments. Accordingly, our experimental hemolytic anemia is not produced by the intravascular hemolysis due to the action of hemolysins and complement. Our experimental hemolytic anemia is the case of a medium dose and coincides well with the hemolytic anemia in guinea pig which Dameshek[1] produced.

When we applied the Coombs' test, we found that the erythrocytes from the rabbits which had received anti-serum continued to be agglutinated as long as their osmotic fragility remained increased. In other words, the complete antibodies disappeared quickly from the circulating plasma of rabbits, but antibodies remained attached to the red cells as long as the osmotic fragility continued high. We therefore supposed that they were incomplete antibodies and responsible for the increased osmotic fragility which continued for about 2 weeks. We proceeded to find out if hemolytic anemia can be produced by the injection of anti-erythrocyte serum which contains incomplete antibodies but no complete antibodies.

There is a difference in thermostability[14] between incomplete antibodies and complete antibodies. It is generally accepted that complete antibodies are heat labile. We heated anti-erythrocytes serum at 65°C for 30 minutes. The activity of hemolysins and hemagglutinin in saline

medium was completely abolished after this process. But we could still demonstrate the existence of incomplete antibodies in this heat-treated anti-serum by means of the indirect Coombs' test.

When we injected intravenously the heat-treated anti-serum into rabbits, we produced increased osmotic fragility, reduction of red cells count, changes in Price-Jones' curve, microspherocytosis and reticulocytosis. Increased bone marrow erythropiesis also occurred. The direct Coombs' test proved as long as the osmotic fragility remained elevated. This means that we can produce the same type of an acute hemolytic anemia by the injection of heat-treated anti-serum as that produced by the injection of non-heat-treated anti-serum which includes complete antibodies.

We next inspected by means of electron microscopy the erythrocytes membranes of rabbits. The normal erythrocytes membranes[15)16)] are thin and show fine granular structure. They have no folds and holes. However, the membranes of red blood cells taken from rabbits receiving the injection of heat-treated anti-serum and the membranes of erythrocytes sensitized in vitro with heat-treated anti-serum lost normal fine granular structure. Coarseness and roughness of the membrane structure and formation of folds and holes were seen. We are not yet in a position to judge what these changes singnify. But we can at least assume that incomplete antibodies attached to the surface of erythrocytes are responsible for these changes.

Spleen and liver showed marked hemosiderosis 24 hours after the injection. The hepatic hemosiderosis regressed within a week, but that in spleen could still be seen 7 days after the injection.

A remarkable shortening of life span of erythrocytes was demonstrated, the rabbits which had received the intravenous injection of heat-treated anti-serum, by means of Cr^{51} tagged erythrocytes transfusion method. However, this shortening of life span of erythrocytes was not demonstrated in splenectomized rabbits which had received the injection of heat-treated antiserum, though they showed the decrease of osmotic fragility of red cells and microspherocytosis.

Judging from these results we think that incomplete antibodies play a significant role in the mechanism of immunohemolytic anemia in rabbits caused by anti-erythrocytes serum injection. In the case of acute immunohemolytic anemia caused in rabbits by the injection of anti-erythrocytes serum, incomplete antibodies in this serum may cover the red blood cells to produce microcytes and spherocytes. Such erythrocytes covered by incomplete antibodies will undergo changes in membrane structure with a resultant increase in osmotic fragility and a reduction of life span. These affected red blood cells may be eventually destroyed in the spleen. Simultaneous with the destruction of the affected erythrocytes,

the bone marrow may be stimulated and show increased erythropoiesis and the reticulocytosis may become evident.

Conclusion

1. The injection of anti-erythrocytes serum brings about an acute hemolytic anemia in rabbits. Increased osmotic fragility of erythrocytes, microspherocytosis and reticulocytosis are observed.

2. Complete antibodies such as hemolysins and hemagglutinins disappear quickly from the circulating plasma of rabbits receiving anti-erythrocyte serum, but antibodies remained attached to the red blood cells as long as the osmotic fragility of erythrocytes continued high.

3. The injection of heat-treated anti-erythrocyte serum which contains incomplete antibodies but no complete antibodies can produce the same type of an acute immunohemolytic anemia as that produced by the injection of anti-erythrocyte serum which contains both complete and incomplete antibodies.

4. By means of electron microscopy, coarseness and roughness of membrane structure and formation of folds and holes are demonstrated in the red blood cells of rabbits receiving the injection of heat-treated anti-serum.

5. A remarkable shortening of the life span of erythrocytes in the rabbits which had received the intravenous injection of heat-treated anti-serum was demonstrated by the transfusion method of the Cr^{51} tagged erythrocytes. However, this shortening of erythrocytes life span was not demonstrated in splenectomized rabbits.

6. A marked hemosiderosis is found in spleen and liver 24 hours after the injection of heat-treated anti-serum. The hepatic hemosiderosis regressed within a week, but that in the spleen can still be seen.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Urinary 17-KS, its Fractions and Urinary 17-OHCS in Diabetes Mellitus with Vascular Complications

By

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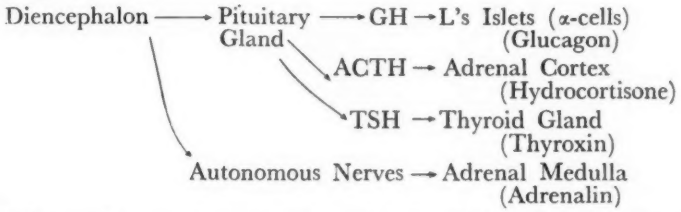
(Received for publication, April 25, 1959)

It is no doubt that diabetes mellitus occurs as a result of deficiency in insulin due to hypofunction of the pancreatic Langerhans' islet cells, but no one nowardays would call diabetes mellitus a disease caused by a mere change in the Langerhans' islets. At present, diabetes mellitus is considered to be due to disturbance of some endocrine organs[1)2)3)].

With recent striking advances in the chemistry of enzymes and hormones, the role of hormones in the metabolism of sugar is becoming more understood. In summary, the presently accepted antiinsulin system is something as follows.


Diencephalon
Pituitary Gland
GH → L's Islets (α-cells) (Glucagon)
ACTH → Adrenal Cortex (Hydrocortisone)
TSH → Thyroid Gland (Thyroxin)
Autonomous Nerves → Adrenal Medulla (Adrenalin)

Hydrocortisone and cortisone act in stimulating the gluconeogenesis from proteins and lipids and inhibiting the sugar utilization at the peripheral areas as glucocorticoids, and are in the closest correlation with sugar metabolism.

Many reports[4)5)6)] on this subject have been made from this Clinic. Many of them showed excitation of the adrenocortical function in diabetes mellitus, this tendency being more marked in the cases with severe diabetes or with complicating diseases. This report concerns the measurements of the urinary contents of 17-KS fractions and 17-OHCS of diabetes mellitus cases with vascular complications.

Experimental

Methods and Materials

The Henry's method[7] as modified by Muraki[6], adding the pressure method into methanol-benzene eluent chromatography was used to measure the urinary 17-KS fractions. The urinary 17-OHCS was measured by Porter-Silber[8], Reddy[9] and Smith's[10] modified method. In the cases of high proteinuria, in which emulsions are easily formed protein was first eliminated with Somogyi's solution. As sugar is apt to interfere with the reaction, preliminary measurements were done with adding various concentrations of glucose. The resultant coloration was not affected at the concentrations of glucose below 5%, but the 17-OHCS content tended to be overestimated at higher concentrations, so that a correction curve was prepared to be applied in the cases with urine sugar of 5% and above.

As control subjects the ambulatory patients with other diseases but free of endocrine disturbances were used. Experimental subjects were diabetic patients treated with Yamakawa-Kurokawa's high-carbohydrate low-fat diet, but before initiation of insulin administration. All of these subjects were selected from the wards.

Results

17-KS Fractions: 15 non-diabetic controls were tested, as shown in Fig. 1. Here, the I+II fractions average 13.6%, fluctuating between 9% and 20%, the III fraction averages 13.6% (9–21%), the IV+V fractions

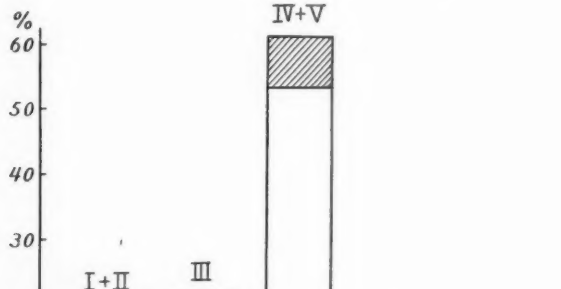


Fig. 1. Urinary 17-KS fractions in non-diabetic control, the shaded area showing the range of fluctuation of the percentages.

average 56.9% (53.6–61.1%), the VI+VII fractions average 10.2% (7–12.6%) and the VIII fraction averages 4.7% (2.0–8.2%). The results of analysis of urine from 15 simple diabetic patients, including 9 men and 6 women ranging from 20 to 67 years of age were as shown in Table I.

TABLE I

Urinary 17-KS Fraction in Diabetic Mellitus

Case	Age	Sex	Fraction (%)					Total 17-KS mg/24 h
			I+II	III	IV+V	VI+VII	VIII	
1	58	M	9.9	14.8	59.0	14.1	2.2	
2	52	M	12.0	19.3	52.3	13.8	2.6	
3	67	M	12.4	24.5	56.3	16.3	10.5	
4	50	M	11.6	16.5	55.8	13.4	2.4	8.0
5	53	M	13.3	23.6	46.9	12.2	4.0	9.7
6	56	M	13.4	16.2	53.4	13.3	3.6	
7	64	F	10.3	13.5	55.4	17.5	4.5	6.2
8	61	F	13.3	13.3	59.1	10.7	3.6	6.0
9	43	F	9.2	18.8	55.4	12.7	3.9	6.4
10	65	F	12.0	15.2	49.0	18.5	5.2	
11	27	F	12.7	15.5	49.1	12.3	5.3	11.4
12	20	M	15.5	16.2	59.4	13.4	4.7	
13	49	M	14.7	10.5	52.5	11.4	10.3	
14	62	M	14.5	14.6	52.5	12.4	6.0	
15	37	F	11.4	19.2	52.4	12.7	4.4	8.1
Mean value			12.8	16.8	53.2	12.4	6.2	

The I+II fractions were within the normal range in all the cases. The III fraction showed high values in the Cases No. 3 and No. 5, while the IV+V fractions were nearly always subnormal and never exceeded the normal. On the other hand, the contents of the VI+VII fractions were never below normal but showed high values in all the cases except the Cases No. 5, 8, 11, 13 and 14. The VIII fraction content was supernormal in the Case No. 13, remaining within the normal range in all the other cases. In short, the changes in the contents of the 17KS fractions may be said as consisting in a decrease of IV+V and an increase of VI+VII fractions. The mean values of the fraction contents of 15 control cases and of 15 diabetic patients are illustrated in Fig. 2. It is seen that in the latter only the I+II fractions remain normal, while the fractions III, VI+VII and VIII tend to be higher and the IV+V fraction to be lower than in the former.

The results of measurement of 17-KS fractions in 10 diabetic cases

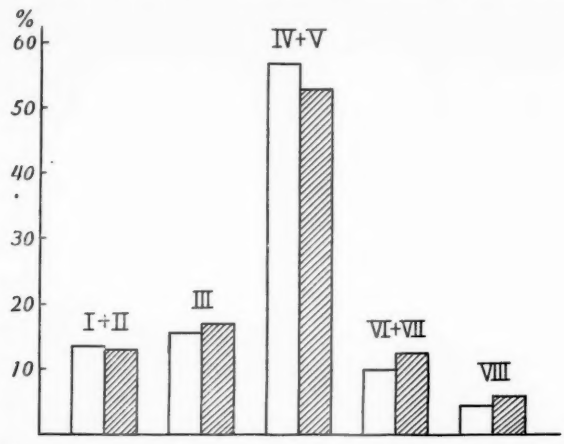


Fig. 2. Urinary 17-KS fractions in controls and diabetic cases (average). The white columns show control, the shaded column shows diabetic cases.

TABLE II

Urinary 17-KS Fractions in Diabetic Cases with Vascular Complications

Case	Age	Sex	Fraction (%)					Blood Pressure	Urinary protein	Retino-pathy
			I+II	III	IV+V	VI+VII	VIII			
1	50	M	11.6	16.5	55.8	13.4	2.7	142- 88	(−)	(+)
2	52	M	12.0	19.3	52.3	13.8	2.6	112- 68	(−)	(+)
3	56	M	13.4	16.2	53.4	13.3	3.6	120- 82	(−)	(+)
4	53	M	13.3	23.6	46.9	12.2	4.0	172- 96	(−)	(−)
5	65	F	12.0	15.2	49.0	18.5	5.2	154- 80	(−)	(−)
6	61	F	13.3	13.3	59.1	10.7	3.6	192- 86	(−)	(+)
7	67	M	12.4	24.5	36.3	16.3	10.5	182-102	(卅)	(+)
8	64	F	10.3	13.5	55.4	17.5	4.5	210-108	(+)	(+)
9	58	M	9.9	14.8	59.0	14.1	2.2	170-110	(+)	(+)
10	43	F	9.2	18.8	55.4	12.7	3.9	165-110	(+)	(+)
Average value in simple diabetic cases			12.8	16.8	53.2	12.4	6.2			
Average value in nondiabetic controls			13.6	13.6	59.6	10.2	4.7			

having vascular complications, including 6 men and 4 women were shown in Table II, and the mean values of 17KS fractions obtained on patients with or without complications are shown in Fig. 3. To see what kind of

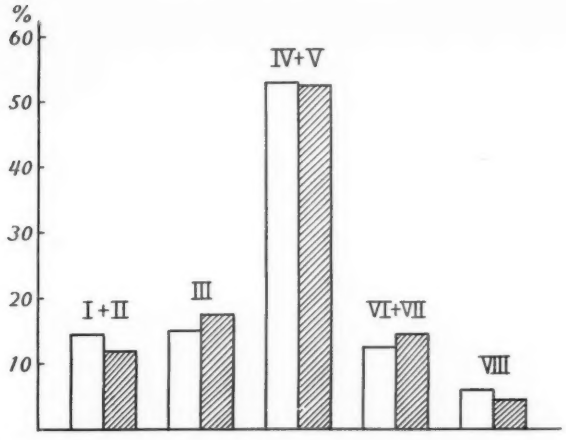


Fig. 3. Urinary 17-KS fractions in diabetic cases with and without vascular complications. The shaded columns show diabetics with complications.

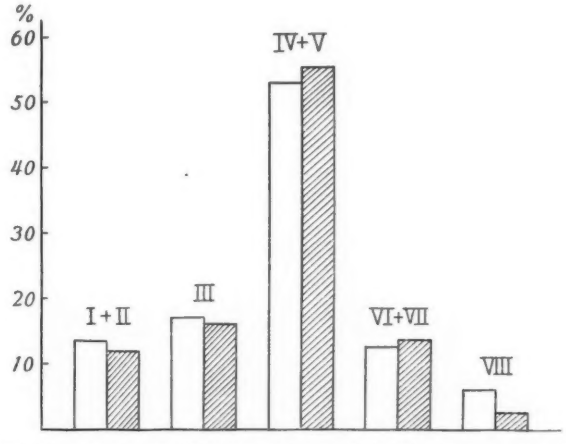


Fig. 4. Urinary 17-KS fractions in case No. 2 (diabetic case complicated with retinopathy), 52 years old male.

complication has the greatest effect on the 17-KS fraction contents, the values in cases with retinopathy (Case No. 2), hypertension (Case No. 5) and nephropathy (Case No. 7) are compared with the mean values in other cases without complications (Figs. 4, 5 and 6). The effect on 17 KS fraction content appears to be the least with retinopathy, and the greatest

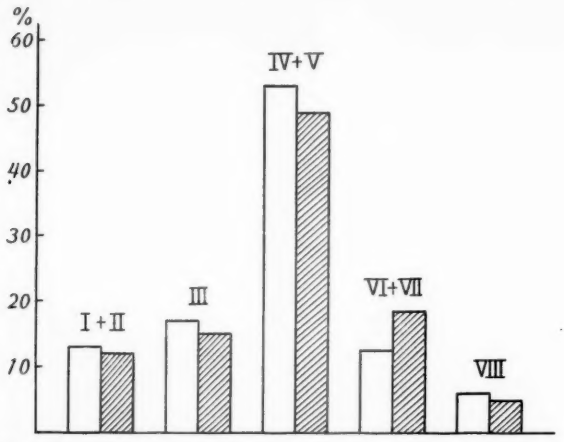


Fig. 5. Urinary 17-KS fractions in diabetic case complicated with hypertension, 65 years old female.

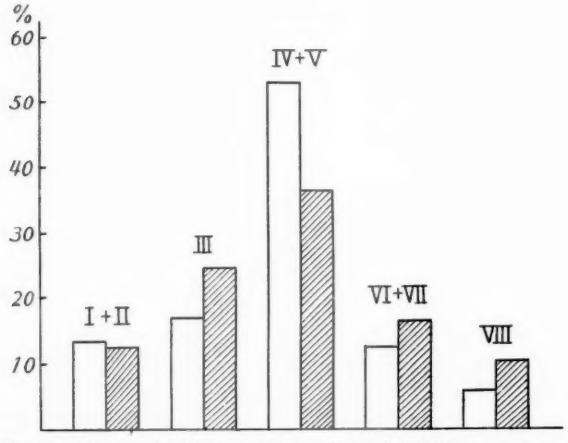


Fig. 6. Urinary 17-KS fractions in Kimmelstiel-Wilson syndrome, 67 years old male.

with nephropathy. In particular, the Case No. 7 showed high grades of hypertension and proteinuria and died soon after the examination, and though the possibility of other factors responsible for such changes is of course not excluded, but at any rate, it is a particularly interesting example.

17-OHCS in urine: Control measurements were made on urine from 93 non-diabetic subjects, including 51 men and 42 women (Table III).

TABLE III

Urinary 17-OHCS in Non-diabetic Controls (mg/day)

Male (51 cases)						Female (42 cases)					
Age	Name	17-OHCS	Age	Name	17-OHCS	Age	Name	17-OHCS	Age	Name	17-OHCS
17	K.K.	4.2	40	T.A.	6.7	17	K.K.	6.8	48	M.O.	7.6
18	K.S.	3.7	40	Y.N.	7.8	19	M.K.	5.5	49	K.S.	7.5
18	N.S.	4.1	41	J. I.	6.1	19	R.T.	3.4	51	H.Y.	6.5
18	S.T.	4.6	41	Y.M.	9.3	20	T.S.	8.5	53	M.M.	7.3
19	K.T.	4.7	42	G.K.	5.4	22	E.S.	8.6	53	M.S.	6.4
19	T.N.	8.9	42	S.O.	6.7	22	Y.S.	6.2	53	M.S.	4.7
21	K.E.	8.1	43	H.H.	8.1	22	K.K.	5.6	54	M.I.	4.2
24	S.K.	6.3	44	S.T.	6.7	23	K.S.	6.5	54	Y.R.	5.5
25	T.K.	6.7	47	S.H.	4.4	23	T.K.	6.2	56	T.S.	7.2
26	S.K.	4.7	47	T.H.	4.8	23	M.O.	5.4	58	N.S.	7.2
28	T.K.	7.6	51	S.S.	5.7	25	K.S.	5.2	58	M.K.	8.1
28	T.S.	7.8	52	Y.K.	5.0	26	Y.T.	6.5	59	U.K.	5.6
28	K.T.	7.6	53	I. B.	8.7	26	Y.Y.	4.2	60	S.H.	5.8
28	K.H.	4.3	54	K.S.	5.7	27	K.H.	5.8	62	S.K.	8.3
29	A.M.	5.6	55	K.H.	6.2	30	T.S.	7.9	65	T.K.	4.8
29	K.K.	5.2	56	Y.O.	7.5	33	K.G.	6.8	66	M.M.	3.2
30	A.K.	6.0	59	M.O.	6.9	33	S.S.	3.9			
30	K.S.	6.1	60	I. I.	5.4	33	R.T.	7.0			
31	K.H.	8.4	61	H.T.	9.3	36	N.K.	5.4			
32	H.S.	6.7	62	R.S.	8.2	37	M.K.	4.2			
33	Y.H.	6.3	64	K.Y.	7.4	39	T.K.	6.8			
33	K.K.	8.6	65	Y.O.	5.4	39	T.I.	5.6			
33	N.O.	6.8	67	Z.T.	4.6	40	S.T.	4.7			
34	T.A.	5.2	67	Y.S.	7.1	40	R.S.	5.4			
34	Y.T.	8.1	69	A.M.	2.7	42	H.Y.	4.1			
34	M.I.	5.2				47	M.S.	6.7			

The content of this compound averaged 6.4 mg./day ranging between 2.7 mg./day and 9.3 mg./day in men, averaged 6.1 mg./day ranging between 3.2 mg./day and 8.6 mg./day in women (a little lower than in men), and in the total of men and women, averaged 6.2 mg./day, ranging between 2.7 mg./day and 9.3 mg./day, as shown in Fig. 7.

The results of similar measurements on urine from 19 diabetic patients are shown in Fig. 8. Here, the value is seen fluctuating in a rather wide range between 2.1 and 25.2 mg./day, the mean value 11.3 mg./day being nearly twice that of the control group.

Now, these 19 diabetic cases comprised 11 cases with and 8 cases with-

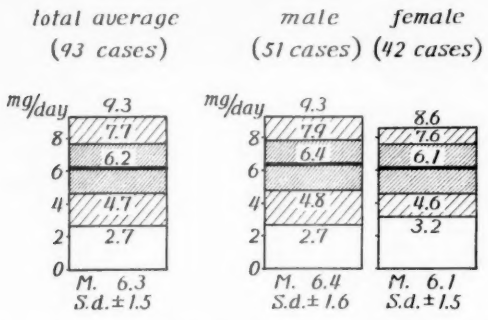


Fig. 7. Urinary 17-OHCS in non-diabetic controls. The faintly schaded area shows the range of fluctuation, the darkly shaded are shows the statistical normalrange, and the dark bar indicates the mean value.
M.=Mean value. s.d.=standard deviation.

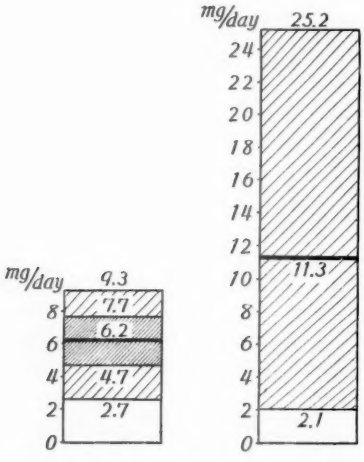


Fig. 8. Urinary 17-OHCS in diabetic cases, showing average values in 19 cases, of which 11 with vascular complications and 8 without complications.

out complications. As shown in Fig. 9, while the diabetic patients free of vascular complications showed the values between 2.1 and 14.8 mg./day, averaging 7.2 mg./day only a little higher than the normal, the cases with complicating disease showed an enormous fluctuation of more than 20 mg./day in their values, ranging between 4.7 and 25.2 mg./day, and the mean value was even higher, rising to 14.1 mg./day. The individual values were as shown in Table IV and V. It is obvious that the cases with complications show wide fluctuation and high values. The individual average

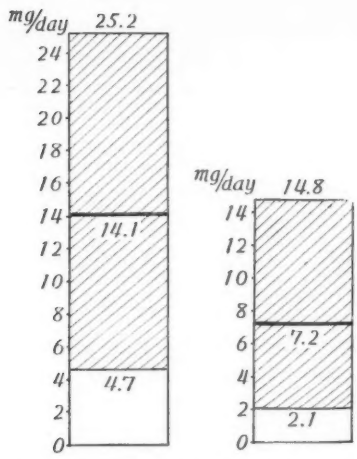


Fig. 9. Urinary 17-OHCS in diabetic cases with and without vascular cimplications. The left column shows urinary 17-OHCS in cases with complications and the right one cases without complications.

TABLE IV

Urinary 17-OHCS in Diabetic Cases without Vascular Complications

Case No	1	2	3	4	5	6	7	8	Control (93 cases)	With complication (11 cases)
Sex. Age	M. 50	M. 49	F. 37	F. 27	M. 7	F. 65	M. 62	M. 20		
Type	I	I	I	I	II	II	II	II		
Mean value	6.8	7.9	9.9	8.0	4.6	5.6	6.7	8.7	6.2	14.1
Upper limit	9.2	9.7	12.7		7.4	11.7	12.2	14.8	9.3	25.2
Lower limit	5.0	6.5	7.1		2.1	3.8	3.0	4.0	2.7	4.7

excretions of urinary 17-OHCS of the diabetic cases with complications are shown in Table VI. Whereas the mean value in diabetic cases without complications is not much different from that in the controls. The value is markedly high in the cases with complications, especially in the cases with retinopathy, albuminuria, hypertension and cardiac symptoms. Case No. 11 (Table V) that registered the highest value 25.2 mg./day had Kimmelstiel-Wilson's syndrome and died soon after the examination. No specific correlation could be established between the types of complications and the 17-OHCS excretion.

Table VII shows the contents of free aceton in blood and of 17-KS

TABLE V

Urinary 17-OHCS in Diabetic Cases with Vascular Complications

Case No.	1	2	3	4	5	6	7	8	9	10	11	Control (93 cases)	Without complication (8 cases)
Sex. Age	M. 50	F. 56	M. 56	M. 52	F. 61	M. 53	M. 42	M. 58	M. 64	F. 43	M. 67		
Type	II	I	I	II	I	II	II	II, K.W.	II, K.W.	II, K.W.	II, K.W.		
Mean value	15.3	10.8	14.6	18.2	8.6	19.9	14.4	10.1	15.8	7.2	21.1	6.2	7.2
Upper limit	17.6	12.6	16.8	22.4	11.9	20.4	17.2	15.0	17.2	10.9	25.2	9.3	14.8
Low limit	13.1	9.0	12.2	11.2	7.2	19.5	12.1	7.4	14.4	4.7	17.4	2.7	2.1
Retinopathy	+	+	+	+	−	−	−	+	+	+	+		
Proteinurie	−	−	±	−	−	−	+	+	##	+	##		
Hypertension	−	−	−	−	+	+	+	+	+	+	+		
Remarks					A.S.		A.F.	M.D.	L.V.H				

A.S. = Arteriosclerosis A.F. = Atrial fibrillation
M.D. = Myocardial damage L.V.H. = Left ventricular hypertrophy

TABLE VI

Urinary 17-OHCS in Diabetic Cases with Vascular Complications

Complications	No. of cases	Average
Retinopathy	9	13.5 mg/day
Albuminuria	5	13.7
Hypertension	7	13.8
Kimmelstiel-Wilson's syndrome	4	13.5
Cardiac failure	4	12.2
I Type of diabetes	7	9.5
II Type of diabetes	12	12.3
Diabetes with vascular complications	11	14.1
Diabetes without complications	8	7.2
Total diabetes	19	11.3
Controls	93	6.2

fractions and 17-OHCS in urine, as measured on 5 diabetic patients with complications. As seen in the Table, 2 of the 3 cases with Kimmelstiel-Wilson's syndrome showed supernormal free aceton level, but the level

TABLE VII

Correlation between Urinary 17-KS Fractions, Urinary 17-OHCS and Free Aceton in Blood

Case No.	Age, Sex	Free aceton in blood	Urinary 17-OHCS	Urinary 17-KS III fraction	Urinary 17-KS VI+VII fraction	Complication
1	50 M	1.25 mg/dl	15.3 mg/day	16.5%	13.4%	Retinopathy
3	56 M	1.01 "	14.6 "	16.2 "	13.3 "	"
9	58 M	0.68 "	10.1 "	14.8 "	14.1 "	K-W Sgnd.
7	67 M	2.96 "	21.1 "	24.5 "	16.3 "	"
10	43 F	6.45 "	7.2 "	16.3 "	12.7 "	"

was within the normal range in the one remaining case and the 2 other cases with retinopathy. The 17-KS fraction content perceptibly changed in all the 5 cases, while the 17-OHCS content was high in the 2 cases with retinopathy and in the one case with Kimmelstiel-Wilson's syndrome.

So, we cannot find apparent parallelism between any of these values and the conditions of patients.

Discussion

In 1952, Dingemanse[11] reported the significance of 8 fractions of 17-KS in urine.

I Fraction: Hydrolytic porducts and pigments
II Fraction: Isoandrostanol-6-one 17
III Fraction: Dehydroisoandrosterone and Isoandrosterone
IV Fraction: Androstèrone
V Fraction: Etiocholanolone
VI Fraction: 11 Hydroxy- and 11 Oxy-Androsterone
VII Fraction: 11 Hydroxy- and 11 Oxy-Etiocholanolone
VIII Fraction: Pigments and unidentified steroids

It has been believed that, I and VIII have no clinical significance, II to increase in the cases of adrenocortical tumors and III to increase perceptibly in patients with adrenogenital syndromes. IV+V represent androsterone and other gonadotropic factors and occupy more than half of the total urinary content of 17-KS. VI+VII, representing the end-products of the glucocorticoids hydrocortisone and cortisone, are of significance in carbohydrate metabolism.

The 17-OHCS content in urine is regarded as reductive product of glucocorticoids and consists mainly of tetra-type compounds, its 3/4 being accounted for by tetrahydrohydrocortisone and is 1/4 by tetrahydrocortisone, other components being in extremely small quantities only. The change in 17-OHCS content, therefore, also is of high significance in carbo-

hydrate metabolism.

Many reports have appeared on the state of function of the adrenal cortex in diabetes cases, but they are not always in full agreement. Lowered adrenocortical function in such cases has been reported by Forbes *et al.*[12], Field & Marble[13], Wolfe & Paschkis[14], Talbot *et al.*[15], while absence of any definite tendency has been reported by Miller & Mason[16]. Ohno[17][18] and Takeda[19] say that the 17-OHCS value tends to rise in the cases of extrapancreatic type diabetes mellitus and it tends to be low in the pluriglandular type cases, while the normal level is maintained in pancreatic type cases. Hattori[5] measured the urinary content of 17-KS in diabetic cases and found supernormal values in 28%, normal values in 61% and subnormal values in 11% of the cases, suggesting a slight tendency to higher values. Joslin *et al.*[20] report that the level of the urinary 17-KS content in juvenile diabetes mellitus is always higher than normal. Fajan & Conn[21] measured the sensitivity to cortisone and ACTH in the lineal members of diabetic patients and inferred that the adrenal cortex of such people is in an activated state. Hayashi[22] followed up the change in the counts of eosinophil cells after insulin injections and infers the state of stimulated function in the adrenal cortex of diabetic cases. Okamoto[23] has found the increase of urinary 17-KS, of VI+VII fractions and urinary 17-OHCS already before the spontaneous occurrence of diabetes in the F_4 offsprings of alloxan-diabetes rabbits, and discussed that the adrenal cortex apparently played an important role in the occurrence of diabetes mellitus. Wallach *et al.*[24] measured the free and conjugated 17-OHCS in the blood plasma of diabetic patients and attributes their increases to acidosis. Our measurement of free aceton in blood, however, failed to reveal any correlation with diabetes.

Concerning the relation between the adrenocortical function and diabetes with complications, Becker[25] and McArther *et al.*[26] observed the accentuated adrenocortical function in diabetic patients with complicating retinopathy or nephropathy. Rifkin *et al.*[27] studied the 17-OHCS excretion in urine, its content in the blood, the ACTH tests and determination of urinary excretion of radioactive hydrocortisone administered intravenously, reporting that nó particular exitation of the adrenal function could be seen in the diabetic patients complicated with retinopathy and nephropathy. Maengwyn-Davis *et al.*[28] measured the 17-OHCS content in urine of diabetic retinopathy cases, and reported higher values in these than in diabetes cases without complications. Shadaksharappa *et al.*[29], upon measuring the excretion of urinary phosphomolybdate-reducing substances and of 17-KS, found 17-KS decreased but phosphomolybdate-reducing substances always increased in 3 cases with Kimmelstiel-Wilson's syndrome, in one of whom hyperplasia of the adrenal cortex was observed

upon autopsy. On the other hand, regarding the renal function and the excretion of steroids, Bongiovani & Eberlein[30] measured the clearance of dehydroisoandrosterone and of androsterone, and pointed out that the clearance of the latter is much larger and that this high clearance of androsterone may be attributed to its larger production and the higher concentration in blood, and thought that the hormone was secreted from the renal tubuli into the excreted urine.

In Japan, Okitsu[31] measured the 17-KS fractions in urine of nephropathic cases and reported that the IV+V or the VI+VII fractions were decreased in many chronic nephritis, while all the fractions were found decreased in its terminal stage or in nephrosis. This finding seems to indicate that there is a close correlation between steroid excretion and the renal functions.

The results of our measurements demonstrated increase of the 17-OHCS and the 17-KS contents in urine and of the VI+VII fractions therein, suggesting stimulation of the adrenocortical function. It cannot be decided, however, whether this hyperfunction is one of the responsible factors for occurrence of diabetes mellitus, as advocated by Okamoto, or it is merely a symptom concomitant with complication of diabetes.

Overeating as well as intravenous administration of glucose are considered to produce stress. Yanai[4] followed up the change in the count of eosinophil cells in the blood after oral administration of glucose, and found an indefinite but slight tendency of reduction. If it is true, we can postulate the following vicious circle would be made and it appears to be an unfavorable to the defense mechanism of the living organism:

High blood sugar → Stimulted corticoid secretion → Lowered sugar utilization → High blood sugar.

In the future, we are planning to do more studies on this problem.

Conclusion

1) We measured the 17-KS fractions in urine of diabetic patients with vascular complications and found the VI+VII fractions increased above normal.

2) The urinary 17-OHCS in such cases also found to be increased.

3) Therefore, it is concluded that the function of the adrenal cortex is apparently accentuated in such patients.

4) No differences were observed among the type of complications.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Evaluation of the Method of Weil-Malherbe and Bone for Determination of Catecholamines in Blood Plasma*

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(Received for publication, May 21, 1959)

The method described by Weil-Malherbe and Bone[1)2)3)] for determination of catecholamines in blood plasma is highly sensitive and rather simple in technique. However, several authors[4)5)] consider that this method is poor in specificity for adrenaline (A) and noradrenaline (NA). On this ground the problem of its specificity was studied.

Methods

The experiments were carried out according to the method of Weil-Malherbe and Bone[1)2)3)] in principle, but most of the reagents and the apparatus used were of domestic production. The technique reported in this paper differed from the original in the following items.

A. Reagents

Ethylenediamine tetraacetic acid (EDTA): (Daiichi Pure Chemicals Co.) EDTA-sodium thiosulfate solution:

1 g. EDTA (4 h) is added to 50 ml. redistilled water, then, titrating NaOH, the pH of the solution is brought up to 7.0. 2 g. of $Na_2S_2O_3 \cdot 5H_2O$ is added and dissolved. Finally the whole solution is made up to 100 ml. with redistilled water. This solution, poured into 20 ml. vials, is sterilized by autoclaving.

Sodium acetate buffer at pH 8.4:

Immediately before use, the pH of 0.2 *M* sodium acetate solution is brought up to 8.4 by adding 0.5 *N* Na_2CO_3 dropwise. Ion exchange resin treatment[1)] is omitted.

Alumina: (Merck, for chromatographic adsorption analysis)

* Presented at the 32nd Annual Meeting of the Japanese Endocrinological Society, April 6, 1959

Acid washed and activated at 300°C.
Ethylenediamine dihydrochloride: (Daiichi)
Redistilled ethylenediamine: (Daiichi, for adrenaline estimation)

B. Procedure

The internal diameter of the column was 6 mm.[10] In chromatographing, pressure was used instead of suction.[3][4] In other points, the original method was followed.

C. Measurement of fluorescence

To measure the fluorescence, a "Microfluorometer, Yagi type" (Shimazu Manufactory) was used. The characteristics of the filters are as follows:

Primary filter: maximal transmission at 436 mμ.
Secondary filters:
a) maximal transmission at 530 mμ.
b) cuts off the wave length below 550 mμ.

Results

A. Preliminary experiments

A linear calibration graph was obtained from pure catecholamine solutions (Fig. 1). The differential estimation of A and NA mixture was

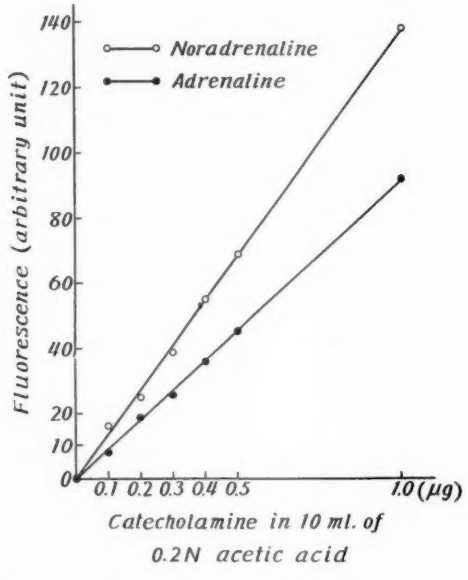

Fig. 1. Calibration graph of catecholamines.

satisfactory (Table I). Recovery of catecholamines added to plasma was 94 per cent on the average (Table II). Four aliquots of plasma were determined separately and the reproducibility was also satisfactory (Table III).

TABLE I

Differentiation of A-NA Mixture in 10 ml. of 0.2 M Acetic Acid

	Content (μg)	Estimated (μg)	Error (%)
A	0.100	0.1013	+1.3
NA	0.100	0.0988	−0.3
A	0.150	0.1510	+0.7
NA	0.050	0.0589	+16
A	0.050	0.0484	− 3
NA	0.150	0.1680	+11

TABLE II

Recovery of Catecholamines Added to 10 ml. of Plasma

Catecholamine added	(μg)	Recovered (μg)	Recovery (%)
A	0.1	0.100	100
NA	0.1	0.080	80
A	0.1	0.106	106
NA	0.1	0.089	89
		Mean	94

TABLE III

Reproducibility of ED Method. 50 ml. Plasma was Divided into 4 Equal Parts, and the Concentrations in Each were Estimated through the Whole Procedure Separately

No. of determination	A (μg/l)	NA (μg/l)
1	0.03	2.4
2	0.27	2.8
3	0.27	2.9
4	0.19	3.2
Mean	0.19	2.88

From these results this method seemed to be excellent. Next, the specificity was tested.

B. Studies on specificity

I. *The relationship between the specificity and the emulsion formation in isobutanol layer*

The plasma eluates obtained by chromatography were condensed with ethylenediamine (ED), and the condensates were extracted into isobutanol by shaking mechanically for 4 minutes, then emulsion formation was always observed in isobutanol layer. The emulsion was never formed when the standards or the reagent blanks were treated in this way. Therefore, the eluates were considered to contain some substances unpurified by chromatography.

The eluate became turbid on adding few drops of sulfosalicylic acid. Quantitation of protein by the method of Nissl-Esbach showed that the eluate contained about 40 μg./ml. protein.

After condensation and extraction of a reagent blank, to which one drop of plasma was previously added, strong emulsion was formed. This emulsion was broken down by centrifuging, and the fluorescence of the supernatant was measured. It was considerably high (corresponding to a mixture of 3.1 mμg. A and 6.6 mμg. NA).

From these results, it seems to be very likely that protein and other unidentified substances which pass through the column unpurified have some influence upon the true catecholamine levels, making them erroneously high.

In order to eliminate the protein, the alumina column was washed before elution with large amount of water. The effluent was collected in fractions of 10 ml. each, for the catecholamines might possibly be washed down by so much water. The fluorescence and the emulsion formation of these fractions and of the eluate after condensation and extraction are shown in Fig. 2. The catecholamines were not eluated by washings, but the emulsion formation was not affected by this procedure. The protein could not be eliminated by this attempt.

II. *Problem of contamination*

A bit of room dust was mixed with the reagent blank. After condensation and extraction this sample fluoresced again considerably. Powder of alumina or fragments of a rubber plug were also tested in this way, and similar results were obtained. The fluorescence observed in these experiments has similar characteristics to that of NA, so it was estimated as NA by differential fluorimetry (the values of A were often negative).

The protein mentioned above is also a contaminating factor. The specificity of the ethylenediamine condensation method (ED method) seems to be seriously disturbed by these contaminating factors.

III. *Comparison with trihydroxyindole method (THI method)*

The eluate obtained from large amount of plasma (ca 50 ml.) was

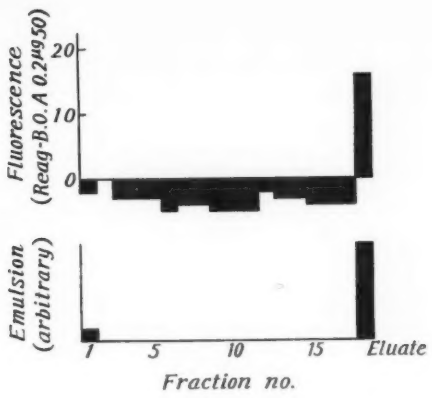

Fig. 2. After the plasma passed through the column, the alumina was washed with 170 ml. water. The effluent was collected in 10 ml. fractions. The fractions and the eluate were condensed, extracted and measured.
Upper: fluorescence.
Lower: emulsion formation.

divided into two parts. The catecholamines in one part were determined with ED method, and in the other with THI (Table IV).

TABLE IV
Comparison of ED and THI Method

Patient		ED (μg/l)	THI (μg/l)
K. K.	A	0.00	0.15
	NA	9.4	0.16
T. G.	A	0.19	0.30
	NA	9.2	0.00
G. M.	A	0.22	0.04
	NA	11.9	0.31

The THI method was carried out according to Price and Price[6], but the principle of different pH (von Euler and Floding[7]) was applied for the differential estimation.

As shown in the table, the values of ED method were significantly higher than that of THI. Taking into consideration the high specificity of the THI method, this marked discrepancy might indicate again the lack of specificity in the ED method.

IV. *Attempt to make an extract blank*

The ED method cannot differentiate the fluorescence due to contamination because it lacks the extract blank. If a sample is divided in two

equal parts, and the catecholamines in one part are destroyed, the latter may be used as an extract blank. In this case, however, it is necessary that the destroying procedure does not give rise to any fluorescence and have no effect on the fluorescence not due to catecholamines.

An attempt for this purpose was made as following: 0.1 ml. of potassium ferricyanide and 1 ml. of 5 *N* NaOH were added to pure catecholamine solutions which were then allowed to stand for 10 minutes, thus destroying the catecholamines in them. These destroying drugs were also added to other identical solutions, which had already been condensed with ED, in order to detect whether or not this procedure had any effect on the fluorescence due to the condensates and to other substances than catecholamines. The results are shown in Table V.

TABLE V

Attempt to Make an Extract Blank. Secondary Filter a) Has a Maximal Transmission at 530 mμ. b) Cuts off the Wave Length below 550 mμ

		Secondary filter	[1]No treatment	[2]Ferricyanide and NaOH added before condensation	[3]Ferricyanide and NaOH added after condensation
I	0.2 N acetic acid 5 ml.	a	0*		−5
		b	0*	8.5	−5
II	ditto	a	100*	−5	−5
	+A 0.2 γg	b	100*	7.5	−3
III	ditto	a	150	−2	−5
	+NA 0.2 γg	b	53	10	−5

* Samples were set at 0 and 100 respectively.

If the fluorescence of the samples in which the catecholamines were destroyed (II_2, III_2) was equal to that of the reagent blank (I_1), and the fluorescence of the samples to which the destructive drugs were added after condensation (II_3, III_3) to that of the samples without treatment (II_1, III_1), then this attempt might be called successful. But, in fact, they differed greatly from each other. This procedure could not be applied for this purpose.

DISCUSSION

Many investigators[2)4)8-13)15)] have estimated the catecholamines in human plasma using the original method of Weil-Malherbe and Bone or slightly modified ones. The average values of the plasma catecholamines in these reports are shown in Table VI.

TABLE VI
Estimated Values of A and NA in Human Peripheral Plasma Using ED Method

Authors		A (μg/l)	NA (μg/l)
Weil-Malherbe and Bone[2]	1953	± 1.18 ± 1.46	± 4.69 ± 3.81
Manger et al.[8]	1954	0.14	3.96
Valk and Price[4]	1956	0.090	2.78
Aronow et al.[9]	1956	0.4	2.1
Gray and Young[10]	1957	0.11	3.4
Kägi et al.[6]	1957	0.28	2.56
Mangan and Mason[11]	1958	0.00	2.09
Weil-Malherbe and Bone[12]	1958	0.2	8.3
Our own data[13]	1958	0.5	5.5

Although the recoveries were reported uniformly as high as 90–100 per cent, a remarkable discrepancy is noted between these average values. It is not acceptable that the true catecholamine levels are so markedly different. The cause of this discrepancy is more easily understood when it is assumed that the samples were purified by the authors in different grade, resulting in different contaminating effects.

Kägi *et al.*[15] noticed that the fluorescence of the blanks was larger when the glasswares used were dried at 120°C than when dried at room temperature. Dust, powder of alumina or fragments of rubber fluoresced considerably if they were added to the reagent blanks. Since protein was found in the eluate, other components of plasma might also be present in it. These substances also fluoresced after determination procedure. All these results indicate that the determined values with ED method are seriously influenced by contamination.

Von Euler[5] found that the major substance determined by ED method in cattle plasma was dihydroxyphenylacetic acid (dopac). Although we did not make any study on this point, it may be the same in human plasma.

Weil-Malherbe and Bone[3] themselves have compared the ED method and bioassay, finding the values of the former were significantly higher than those of the latter.

Valk and Price[4], comparing the ED and the THI method, reported similar results to ours.

The THI method is considered to be specific for active catecholamines, A and NA, even in the presence of other catechol compounds.[7] Further, it eliminates the effect of contamination by preparing the extract blanks. The values estimated with THI method by various authors[4]

[6][13][16] are rather uniform, and in accord with the values assayed biologically[14] (Table VII). Therefore, the higher values obtained by ED method than by THI are considered to be due to the lack of specificity in the former.

TABLE VII

A and NA Concentrations in Human Peripheral Plasma Determined by THI or Bioassay

Authors		Method	A (μg/l)	NA (μg/l)
Lund[16]	1951	THI	<1.0	<1.0
Holzbauer and Vogt[14]	1954	Bioassay	<0.06	<1.0
Valk and Price[4]	1956	THI	0.00	0.20
Price and Price[6]	1957	THI	0.01	0.34
Our own data[13]	1958	THI	0.16	0.16

Even in the ED method, the effect of contamination may be eliminated if an extract blank is available. Though the attempt to make an extract blank failed, it may be achieved by further investigation. In that instance, however, since one half of the sample must be used as the extract blank, the sensitivity is also halved, approaching to that of THI method. For this reason such an attempt seems to be useless.

In the THI method a large amount of blood is necessary because this method is not sensitive enough. The ED method seems to have a high sensitivity even at such low concentrations as in human plasma, but it is only because A, NA and other catechol derivatives together with contaminants are all measured in this method. A micromethod is desirable which permits accurate estimation in small amounts of plasma, but none is currently available.

SUMMARY

The method described by Weil-Malherbe and Bone for the estimation of the catecholamines in blood plasma was reinvestigated. The values estimated by this technique are unduly high because protein and possibly other contaminants are present in the eluate. When compared with THI method, the ED method gives significantly higher values than the former.

Attempts were made to eliminate protein in the eluates and to make an extract blank by destroying the catecholamines in one aliquot of the sample, but both failed.

The remarkable discrepancy between the catecholamine levels estimated by various authors using ED method is easily understood if it is

assumed that the samples were purified in different grade, resulting in different degrees of contamination.

The THI method may be excellent one, but it requires large amounts of blood for estimation, because it is not adequately sensitive. Thus the THI method is also unapplicable in clinical laboratories.

Acknowledgement

We are indebted to Prof. Dr. Tatsuo Torikai for his kind advice and continuous leadership throughout this study.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Locus of the Action of Gamma Aminobutyric Acid in the Suppression of Muscular Movements in Cats

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(Received for publication, May 25, 1959)

Introduction

Among various actions of γ-aminobutyric acid (GAB) upon the activity of the central nervous system[1)2)3)], the most remarkable one is an anticonvulsant action which was first noted by Hayashi[4)]. He reported that GAB, when administered to an animal by various means, could suppress muscular movements elicited by convulsant drugs which were injected into the systemic circulation or applied directly to the motor cortex. Studying actions of GAB and related substances in comparison with those of Florey's Factor I, McLennan[5)] could confirm the suppressing effect of GAB upon convulsive movements, and supposed that this effect might be exerted upon cortical neurones themselves.

The experiment to be reported here was done to see what part of the central nervous system was involved in the motor suppression following intracisternal injection of GAB. The results so far obtained suggest to us that the site of action of GAB in the motor suppression is located most probably at the reticular formation of the brain stem.

Experimental

Method

Cats, weighing 1.5 to 4 kg, were used. Surgical operations, such as tracheal cannulation, exposure of the cerebral cortex, laminectomy and so on, were carried out under an initial anesthesia produced by an intraperitoneal injection of pentobarbital sodium (30 mg/kg of body weight). Experiments were conducted maintaining a constant level of anesthesia by intermittent intravenous injections of small doses of the same anesthetic.

For recording electrical activity of the cerebral cortex, a silver wire with a balled tip was gently placed upon the exposed cortical surface, and

it was used as a different electrode against an indifferent one placed on inactive tissues. Electromyographic activities associated with movements of the legs were recorded by inserting needle electrodes into suitable muscles. In some experiments, impulse discharges were recorded from ventral roots of the spinal cord by hooking up the root with two silver wires separated by several millimeters. For amplifying the electrical activities, an ink-writing electroencephalograph (Sanei) and a double beam cathode-ray oscillograph in conjunction with suitable preamplifiers were used. For electrical stimulation we employed an electronic stimulator with an independent control of stimulus parameters.

GAB, for the use of intracisternal injection, was made up of 2 to 10% solution in a physiological saline.

Results

1) Effect of intracisternally injected GAB upon cortically induced movements

To produce cortically induced movements in one foreleg, 0.5% strychnine was applied to the foreleg area of the contralateral motor cortex. For the purpose of preventing spread of the applied solution, a small leucite ring was held with a light pressure on the cortical point to be treated and a few drops of the solution were poured inside the ring.

Several minutes after the strychninization, there developed spikes at the treated cortical point, and they were accompanied with muscular contractions in the foreleg. Though there was a considerable fluctuation in the amplitude of EMG response, an exact one-to-one correspondence could be ascertained between the cortical and the peripheral event (record 1 in Fig. 1). Two minutes after an intracisternal injection of 8 mg. of GAB, there was found a slight reduction in the motor response (record 2). As the time elapsed, the motor suppression became manifest gradually and its maximum was attained about 8 minutes after the injection (record 3). It was found about 12 minutes later that the motor response could appear with about the same amplitude as found before the injection (record 4).

As to electrocortical activity, it is worth noticing that, while the motor response was brought to a strongly suppressed state, spikes of the motor cortex were maintained at an approximately constant level of activity. Since it has been established that, when GAB is applied directly to the strychnine-treated cortex, there is a characteristic modification of the strychnine spike[2], the above-mentioned results convince us that intracisternally injected GAB did not reach the motor cortex with any appreciable concentration. If we are allowed to regard the spike activity recorded from the cortical surface as a measure for the process of triggering peripheral

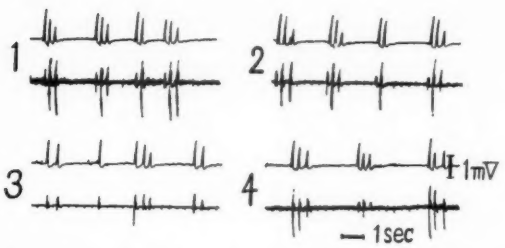

Fig. 1. Strychnine spikes of motor cortex and EMG responses of contralateral foreleg. 1 : Control before injection. 2 : 2 minutes after injection of 8 mg of GAB. Motor responses were slightly reduced. 3 : 8 minutes after injection. Motor suppression was maximal. 4 : Recovery at 20 minutes. During the whole course of motor suppression, spikes at cortex remained unchanged. Noisy baselines of EMG were due to shivering. It was suppressed in 3.

muscular contractions, we are led to the conclusion that a suppression of the cortically induced movement is caused by GAB without making any serious disturbance in the cortical neuronal mechanism which controls the peripheral motor response. In this sense, McLennan's supposition can not be accepted

Another experiment in the same line was done taking as indices a foreleg movement due to electrostimulation of the motor cortex, and cortical afterdischarges recorded from the stimulated point following each stimulus. A time course of the motor suppression after an intracisternal injection of 7 mg. of GAB was illustrated in the inset of Fig. 2, where mean EMG amplitudes taken from the several consecutive responses were plotted against time after the injection. As was observed in the experiment of Fig. 1, electrical activity of the motor cortex in response to electrostimulation remained intact, though the motor response suffered a marked suppression. This will be seen most clearly in sample EEG records of Fig. 2 which were taken simultaneously with EMG responses.

There is an additional finding in the experiments mentioned above. A phenomenon of spontaneous shivering, which is most common in a cat lightly anesthetized with barbiturate, was also found to be suppressed by intracisternally injected GAB. This feature will be seen from the records of Fig. 1 and 2, where the baseline of the EMG records appears somewhat noisy if the animal shows shivering. In this regard, we have had the impression that a suppressing effect of GAB appears earlier and lasts longer with the shivering than with the cortically induced movement. Since the shivering was found to be a sensitive indicator for judging the effect of GAB, we monitored EMG activity of shivering as a routine procedure

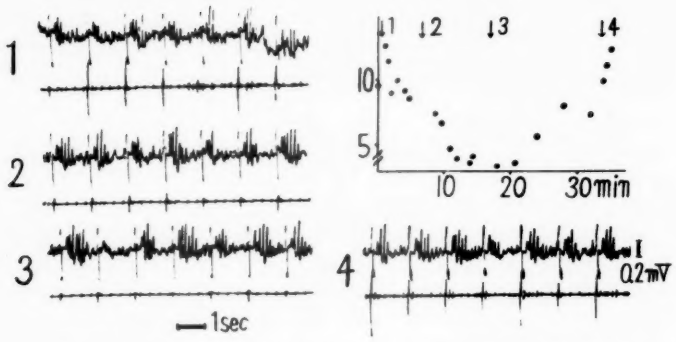

Fig. 2. Cortically evoked EMG responses and cortical afterdischarges. 1 : Control before injection. 2, 3 & 4 : 7, 17 & 35 minutes after injection of 7 mg of GAB. While motor response passed through a strong suppression, cortical afterdischarge following each stimulus suffered no change.

Inset. A time course of motor suppression. Mean amplitudes of several consecutive EMG responses were plotted in arbitrary unit against time after injection. Arrows marked by numbers indicate times at which illustrated sample EEGs and EMGs were taken.

in all the experiments carried out.

2) Suppression of spinal reflexes

Since it was found that the suppression of the cortically induced movement following intracisternal injection of GAB was not an event occurring at the cortical level, one might suppose that the substance so introduced would flow down within the subdural space and reach the spinal cord to suppress the activity thereof, resulting in a failure of the impulses from the motor cortex to cause a ventral root discharge. If so, the spinal cord activity, when examined by taking a reflex discharge as index, will also be suppressed by applying GAB directly to the exposed cord. However, this is not the case. We found that a ventral root discharge in response to single shock stimulation of a dorsal root remained unaffected when the relevant spinal segments were immersed for a long period in a solution of GAB with a considerably high concentration. A similar finding has also been reported by McLennan.

Though GAB, directly applied to the spinal cord, does not affect the spinal reflex activity, it does so if administered through the intracisternal route. In this case the suppression of the spinal reflex is found to proceed in much the same way as is observed with the cortically induced movement. This can be seen in Fig. 3, where a cortically induced movement and a reflex movement, both produced in the same foreleg, were recorded alternately under the action of GAB. Here, the cortically and the reflexly

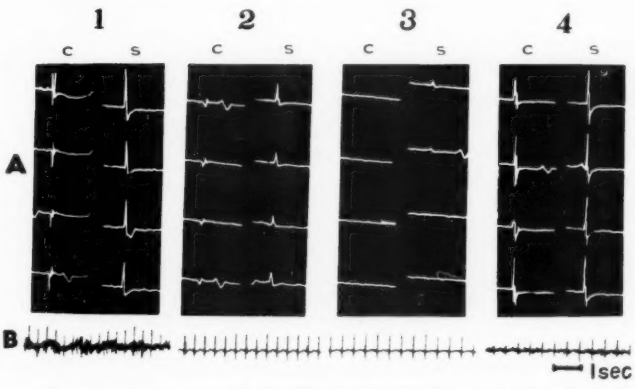

Fig. 3. EMG records of cortically evoked movements (C) and of reflexly evoked ones (S) in the same foreleg. Four consecutive responses were shown for each type of movement. 1 : Control before injection. 2, 3 & 4 : 1, 10 and 18 minutes after injection of 10 mg of GAB. Extent of shivering at respective stages is seen in EMG records mounted below each frame. Sharp deflections in records of shivering are due to electrical activity of heart.

evoked movement were produced by stimulating, respectively, the contralateral motor cortex and the skin of the forepaw. Following an injection of 10 mg. of GAB, both types of movement suffered simultaneously a marked suppression within a short time after the injection (record 2 and 3), and finally they showed a complete recovery at about the same time (record 4).

The results presented in Fig. 4 came from an experiment where reflex movements in one foreleg and in one hindleg were examined alternately when injecting 10 mg. of GAB intracisternally. In this figure, mean amplitudes of the EMG response obtained with each leg were represented by rectangles for successive stages in the action of GAB. Since there can be found no marked difference between the foreleg and the hindleg in the extent of reflex suppression, one can suppose that the suppressing effect of GAB is exerted upon the spinal reflex without regard for the spinal segment involved.

While the spinal reflex used as test object in the experiment of Fig. 3 and 4 belonged to the type of flexion reflex which is executed through a multisynaptic pathway, a reflex activity studied in the experiment of Fig. 5 was a monosynaptic reflex discharge which was elicited in L_7 ventral root with a latency less than 2 milliseconds by single shock stimulation of L_7 dorsal root. With this type of reflex activity too, the progress of the suppressing action of GAB was much the same as was found with the multisynaptic reflex.

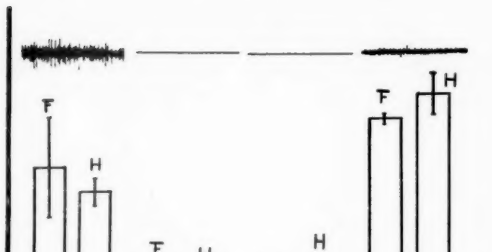

Fig. 4. Reflex responses in foreleg (F) and in hindleg (H). Mean EMG amplitudes are shown by rectangles, each carrying at its top a line representing twice the standard error of mean. A: Control before injection. B, C & D: 4, 7 and 38 minutes after injection of 10 mg of GAB. At top, EMG records of shivering were presented for successive stages.

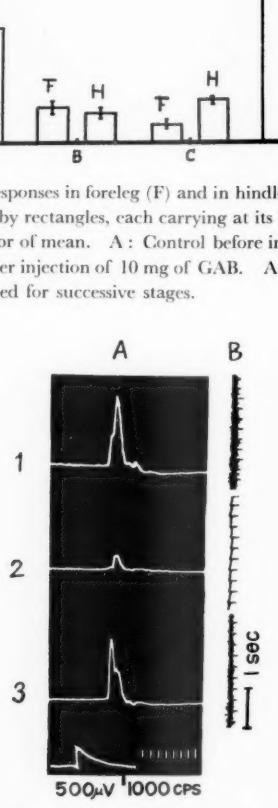

Fig. 5. Monosynaptic reflex discharge. A: Reflex discharge from L_7 ventral root. B: EMG records of shivering. Time of recording was before (1), and 5′ (2) and 21′ (3) after injection of 10 mg of GAB.

3) Experiments on decerebrate and spinal animals

With a cat decerebrated at the intercollicular level, the effect of GAB was tested upon a flexion reflex of the leg which was produced in the same way as in the preceding experiments. Within about 15 minutes after spraying 10% solution of GAB around the cut end of the brain stem, we

were successful in demonstrating a suppression of the flexion reflex as clearly as in a normal cat.

In connection with this experiment, an experiment must be mentioned which was done in a cat with the spinal cord transected acutely at lower thoracic segments. As in the experiment of Fig. 4, reflex movements were evoked in a foreleg and a hindleg respectively. An intracisternal injection of GAB, in about the same amount as used in the foregoing experiments, could suppress the reflex movement in the foreleg. However, no sign of the reflex suppression whatsoever could be found in the hindleg.

Based upon the evidence obtained in these experiments, a conclusion can safely be reached that, in order for intracisternally injected GAB to suppress the spinal reflex, the spinal cord must keep a neural connection with a suprasegmental structure which can be limited to the part below the intercollicular level.

4) Ascending influence upon electrocortical activity

In some experiments where cats were very lightly anesthetized and EEG activity of the cerebral cortex consisted of low voltage fast waves, GAB was found to shift EEG activity from a desynchronized state to a synchronized one.

The records mounted in Fig. 6 show that such an ascending effect

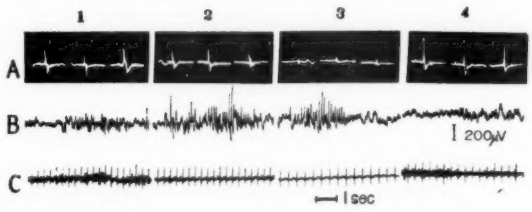

Fig. 6. Simultaneous recording of spinal reflex of foreleg (A), EEG (B) and spontaneous shivering (C). 1: Control before injection. 2, 3 & 4: 3, 10 and 28 minutes after injection of 10 mg of GAB. Suppression of spinal reflex and of shivering was associated with synchronized EEG.

of GAB upon EEG is always associated with the suppression of the spinal reflex. In this experiment, electrical activity of the cerebral cortex was observed simultaneously with reflex movements of a foreleg occurring upon stimulation of the forepaw's skin. As soon as 3 minutes after an intracisternal injection of 10 mg. of GAB, there occurred, in association with a suppression of the spontaneous shivering, an EEG deactivation in such a manner that activity of fast waves was definitely reduced and the frequency of occurrence of the spindling markedly increased (record 2). While a synchronized EEG pattern was maintained as before, motor suppression

became most manifest 10 minutes after the injection (record 3). A replacement of a synchronized EEG by a desynchronized one was accomplished almost simultaneously with a recovery of the reflex movement (record 4).

Discussion

It has been reported by Takahashi and his associates[6] that GAB has a strong effect in causing a fall in blood pressure when it is administered either intravenously or intracisternally. Furthermore, these workers have succeeded in showing an instantaneous pressure fall by microinjecting a minute quantity of the substance into the bulbar area which has been classically defined as "depressor point". Though such is actually the case, we do not believe that such motor suppression as demonstrated in the present experiment is a phenomenon secondary to a blood pressure fall in the systemic circulation. A reason for this statement can be found in the experimental evidence presented in the above section. Briefly, in a cat with the spinal cord transected at lower thoracic segments, the reflex suppression due to GAB occurred only in the foreleg, not in the hindleg. This fact must mean that the suppressing effect of GAB upon the spinal reflex is mediated through an intramedullary neural pathway.

We have pointed out that a suprasegmental structure lying below the intercollicular level is most essential for the suppression of the spinal reflex following intracisternal injection of GAB. It seems most reasonable to consider as a candidate for this structure the brain stem reticular formation, which has been found by Magoun and his colleague[7][8] to exert a regulatory influence upon the spinal reflex as well as upon the cortically induced movement. A support for this view can be provided by the experimental finding that, if there is a motor suppression due to GAB, the cortically induced movement and the reflex movement are affected indiscriminately. Among the possible explanations of our findings in terms of activity of the reticular system, the one which we are inclined to take is that there must be at least an inhibition of the reticular facilitatory system. In following this notion, it is very conceivable that an EEG synchronization is associated with a suppression of the spinal reflex, because the ascending EEG activating system and the descending facilitatory system have been supposed to be mostly synergic[9].

Experimenting on the stretch receptor neurone of the crayfish, Kuffler and his collaborator[10] revealed that GAB acted upon the neurone merely as a depolarizing agent, rather than as an inhibitory transmitter substance. If this view can be extended to our findings, an inhibitory effect of GAB upon the reticular facilitatory system should be considered as unspecific. In fact, the motor suppression observed in the present experiment is quite

difficult to be distinguished in its main aspect from a general motor suppression caused by central depressant drugs such as barbiturate. Furthermore, it is to be noted that the effect of EEG deactivation is common between GAB and barbiturate. Such similarity in physiological actions between GAB and barbiturate seems to be consistent with a view that both drugs have a stronger affinity for the reticular formation than for remaining nervous structures.

Summary

A suppressing effect of γ-aminobutyric acid (GAB) upon cortically or reflexly induced movements was studied with cats by injecting the substance intracisternally in amount less than 10 mg.

1. Cortically induced movements due to strychninization or electrostimulation of the motor cortex were arrested by GAB within a short time. At this time there could not be found any noticeable changes of electrical activity in the motor cortex which was the impulse origin of the suppressed motor response.

2. A suppression of spinal reflexes was obtained by administrating GAB. It was associated with a suppression of the cortically induced movement and spontaneous shivering. The reflex suppression was found to occur in decerebrate cats as well, when GAB was sprayed around the cut end of the brain stem.

3. When the spinal cord was transected acutely, the reflex activity of the isolated spinal segments escaped the suppressing effect of GAB.

4. It was found in some cases that EEG synchronization was caused by GAB in association with the reflex suppression.

5. Concerning the site of action of GAB, a supposition was made that the motor suppression due to GAB might be the result of an inhibition of the reticular facilitatory system of the brain stem.

The authors are indebted to Dr. Tomoaki Asano for his invaluable suggestions in regard to preparing the manuscript. A part of the expense for this work was defrayed by a grant of the Ministry of Education.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Cytochrome C and Nucleic Acids in Various Parts of the Dog Heart

By

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(Received for publication, May 25, 1959)

Introduction

The contents of cytochrome C and nucleic acids (NA) in various parts of the dog heart (the auricle, the ventricular septum, the free wall of the right ventricle and the endocardial and pericardial parts of the left ventricle) were estimated in order to see the correlation with the metabolic behaviors[1] in those parts.

Experimental

Method

The experiments were performed in two series.

In the first series, the strips weighing 300 mg. were excised from various parts of the puppy hearts and kept in a buffered saline at 5–10°C for variable intervals of time (ranging from 1 to 100 hours or more) and then homogenized.

In the second series, the pooled samples of the powders of dog heart strips were employed. For this purpose, the strips were excised from the hearts of puppies (1–6 kg.) and adult dogs (10–15 kg.), then dried at 5–15°C and stored in a desiccator for 2 years before the use. Thus, the pooled powders were obtained from 27 dogs of which puppies were 20, as indicated in Table I. The auricles from adult dogs and puppies were combined because of the small amounts.

The **cytochrome C** content was determined spectrophotometrically according to the method of A. Prader & A. Gonella.[2] The stripped samples weighing 1.0–1.5 gm. (a) were homogenized together with 1.25–2.0 ml. of 1 *N* H_2SO_4 solution in the Potter-Elvehjem homogenator, left standing for 20–30 minutes with addition of 0.5–0.75 ml. of 2 *N* NH_4OH solution and then centrifuged at 4000 r.p.m. for 20 minutes.

For experiments of the second series, 400 mg. of the pooled powders

TABLE I

The Pooled Powders (gm., dry weight) Obtained from Various Parts of Dog Heart

Body weight of dog (kg) ↓ / Material from→	Auricle	Ventricular septum	Right ventricle	Left ventricle	
				Endo-cardial parts	Peri-cardial parts
1— 6 (Puppies)	2.0	3.0	3.1	11.2	10.7
10—15 (Adult dogs)	1.1	7.5	8.9	7.0	8.0

were used and calculated as 2.0 gm. of wet weight. The wet weight of the used samples, the volume of the centrifuged homogenates and the supernatants were conveniently represented as a, b and c, respectively.

Allowing the supernatants to stand for 10 minutes at 50°C after addition of the same volume of 50% Ammonium sulfate solution, the impurities such as hemoglobin were filtered away. Addition of a tenth volume of 90% Trichloracetic acid solution (TCA) to the filtrate, followed by standing for 12 hours or more at 5–10°C, caused brown precipitates of cytochrome C. After centrifugation the precipitates were dissolved to 10 ml. (d) with 0.5 *N* NaOH solution, to which 10 mg. or less of $Na_2S_2O_4$-powders were added just before the analysis. The absorption bands in the spectrum of this solution were observed at the socalled Soret-band (415 mμ), 520 and 550 mμ, as showed in Fig. 1, using the spectrophotometer of Hitachi's EPU-2A type. The concentration of cytochrome C (mg. % wet weight) was calculated as follows:

$$\text{Cytochrome C mg. \% wet weight} = (E_{550} - E_{560}) \times 55.6 \times \frac{d \cdot b}{a \cdot c}$$

E_{550} and E_{560}: optical density, measured in 10 mm. thick cubette at 550 and 560 mμ, respectively, in the range of less than 0.5 mμ of the effective band width. These determinations contained the errors of 9% for the strips and 3% for the powders.

The **nucleic acids** were extracted principally according to W. C. Schneider[3] and were analysed by the colour reactions of the HCl-Orcin reaction[4] for Ribose Nucleic acid (RNA) and the Diphenylamine reaction[5] for Desoxyribose Nucleic acid (DNA), using the spectrophotometer.

The strips weighing 300 mg. were homogenized with 8–10 ml. of 5% TCA solution, heated in a water bath of 90°C for 15 minutes and then centrifuged. The precipitates were once more extracted. The combined extracts were adjusted to 25 ml. and analysed. The concentrations of

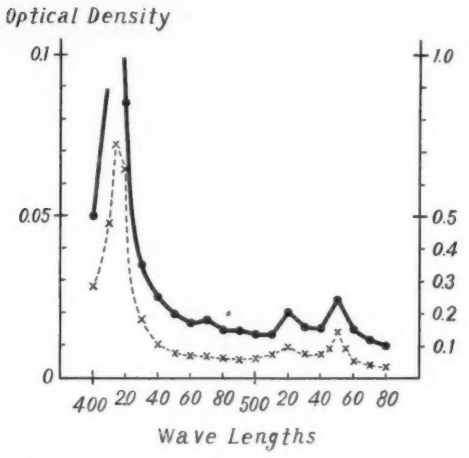

Fig. 1. Absorption spectra of cytochrome C. extracted from the dog heart samples.
Solid line: fresh strips, dotted line: aged powders.
Ordinates: Optical density, the right for the aged powders, the left for the fresh strips.

DNA and especially RNA might be overestimated because the extraction by the ice-cold TCA solution were excluded.

In experiments of the second series, 300 mg. of the pooled powders were extracted by 6 ml. of ice-cold 10% TCA solution in the centrifuge tube and centrifuged. This extraction was once more repeated. The combined supernatants were adjusted to 25 ml. and called as cold extracts. The precipitates were heated with 8 ml. of 5% TCA solution in a water bath of 90°C for 15 minutes and then centrifuged. This procedure was carried once again. The supernatants were combined, adjusted to 25 ml. and called as hot extracts. Each aliquot (2 ml.) of both the cold and the hot extracts was analysed by the above-mentioned colour-reactions. The colour intensity was measured in 10 mm. depth at 610 mμ for RNA and 570 mμ for DNA (the effective band width, less than 1 mμ).

The RNA determination contained the errors of 4% in the excised strips and 3% in the pooled powders.

The errors of the DNA determination were 14% in the case of the strips and 3% in the case of the powders.

The phosphor content in nucleic acid of the samples (NA-P mg. per 100 gm. wet weight) was calculated conveniently as follows, correcting by 10/13 the values of optical densities which were determined as equivalent to 1 γ of NA-P by W. C. Schneider.[3)]

	Optical density, equivalent to 1 γ of NA-P	
	at 610 mμ	at 570 mμ
DNA	0.0128	0.0172
RNA	0.104	0

Results

Figs. 2, 3 and 4 showed the changes of cytochrome C-, DNA- and RNA- contents in the excised strips of the dog hearts during the survival *in vitro*.

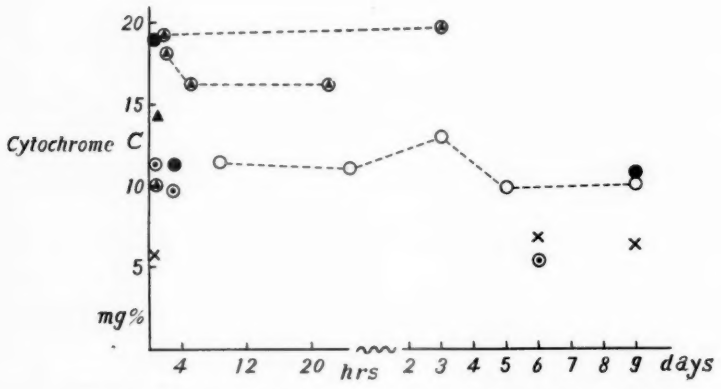

Fig. 2. Changes of cytochrome C contents in the surviving dog heart strips.
× : auricle, ⊙ : free wall of right ventricle, ▲ : ventricular septum, ○ : endocardial parts of left ventricle, ● : pericardial parts of left ventricle.
The values of samples obtained from the same heart are indicated by the dotted lines.
The same notations are used in Figs. 3 & 4.

The fall of cytochrome C contents is observed only in the earlier stage, if it occurs.

It is seen that the DNA contents remain constant but the RNA contents (containing Ribose-necleotides and/or -nucleosides) continue to decrease.

Judging from (1) the initial changes as observed in the case of RNA and cytochrome C, (2) the probable variations of the strip samples, and (3) the experimental errors, the small numbers of measurements cannot differentiate significantly one another about the cytochrome C and NA contents of the freshly excised strips from various parts of the hearts.

This problem may be dissolved to some extent by experiments of the

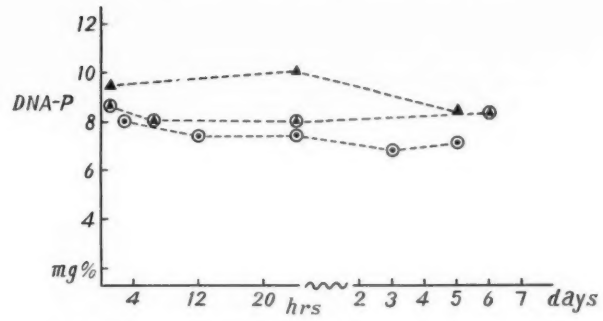

Fig. 3. Changes of DNA contents in the surviving dog heart strips.

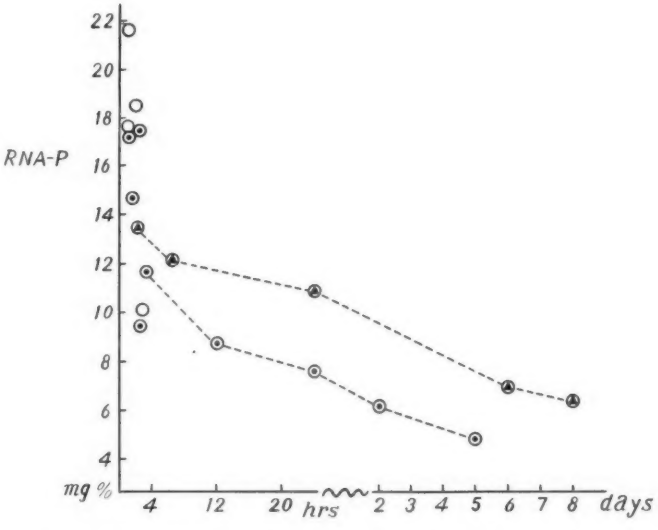

Fig. 4. Changes of RNA contents in the surviving dog heart strips.

second series, *e.g.*, analysis of the pooled powder samples.

In the Tables II, III and IV were indicated the results of the aged powder samples.

The DNA contents in the free walls of the left ventricle, especially, the endocardial part are much less than that in the other parts. However, these differences are not distinguished among those samples from dogs of 10–15 kg. body weight which contain much less DNA than the samples from puppies.

The contents of non-DNA-nucleotides and/or -nucleosides in the

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TABLE II

Cytochrome C Contents in the Pooled Powders of Various Parts of Dog Heart

Cytochrome C content, mg. % wet weight

Body weight of dog (kg) ↓ / Material from→	Auricle*	Ventricular septum	Right ventricle	Left ventricle: Endo-cardial parts	Left ventricle: Peri-cardial parts
(A) 1—6	3.1	14.7	7.4	19.5	14.8
	3.4	13.6	7.7	18.1	14.4
	3.3	**14.2**	**7.6**	**18.8**	**14.6**
(B) 10—15		10.3	6.5	13.5	9.0
		10.4	6.2	12.7	8.0
		10.4	**6.4**	**13.1**	**8.5**
Degree of decrement of cytochrome C (Ratio A:B)	——	**1.4**	**1.2**	**1.4**	**1.7**

* The combined samples of puppies and adult dogs were used.

TABLE III

DNA Contents in the Pooled Powders of Various Parts of Dog Heart

Body weight of dog (kg)			Auricle*	Ventricular septum	Right ventricle	Left ventricle: Endo-cardial parts	Left ventricle: Peri-cardial parts
1–6	E_{570}	Cold extracts	0.0101	0.0044	0.0097	0.0065	0.0093
		Hot extracts	0.1119	0.1085	0.1198	0.0894	0.0904
			0.1124	0.1068	0.1195	0.0851	0.0975
			0.112	**0.108**	**0.120**	**0.087**	**0.094**
	A) DNA-P, mg % wet weight		**5.4**	**5.2**	**5.8**	**4.2**	**4.6**
10–15	E_{570}	Cold extracts		0.0123	0.0150	0.0119	0.0079
		Hot extracts		0.0773	0.0742	0.0670	0.0670
				0.0706	0.0670	0.0721	0.0706
				0.074	**0.071**	**0.070**	**0.069**
	B) DNA-P, mg % wet weight			**3.6**	**3.4**	**3.4**	**3.4**
Degree of increment in cell size (Ratio A:B)			——	**1.5**	**1.7**	**1.2**	**1.4**

* The combined samples of puppies and adult dogs were used.

TABLE IV

RNA Contents in the Pooled Powders of Various Parts of Dog Heart

Body weight of dog (kg)			Auricle*	Ventricular septum	Right ventricle	Left ventricle	
						Endo-cardial parts	Peri-cardial parts
1–6	E_{610}	Cold extracts	0.3017	0.4576	0.3622	0.3884 0.3808	0.3031 0.3075
		Hot extracts	0.3215 0.3002 **0.311**	0.3936 0.4225 **0.408**	0.3645 0.3420 **0.353**	0.4157 0.4012 **0.409**	0.3691 0.3788 **0.374**
	A) RNA-P, mg % wet weight		**1.8**	**2.6**	**2.1**	**2.8**	**2.4**
10–15	E_{610}	Cold extracts		0.3406	0.3346	0.3436 0.3200	0.3986 0.4035
		Hot extracts		0.3410 0.3360 **0.339**	0.3125 0.2857 **0.299**	0.3770 0.3830 **0.380**	0.3605 0.3410 **0.351**
	B) RNA-P, mg % wet weight		——	**2.3**	**2.0**	**2.6**	**2.4**

* The combined samples of puppies and adult dogs were used.

samples from puppies do not exceed 10% of the DNA contents but in the samples of adult dogs much larger.

The RNA contents in the powder samples, though only about 10% of the truly probable values in the freshly excised samples and also independent upon the body weight of dogs, are retained much more in the left ventricle than in the auricle and the free wall of right ventricle.

The non-RNA-nucleotides and/or -nucleosides in any powder sample are contained equally to the RNA in the term of optical density of the colour reaction.

Figure 1 shows that the same cytochrome C as that of freshly excised samples is extracted from aged powders. And the concentration of cytochrome C in the aged powders are surprisingly kept as well as in the freshly excised strips. Cytochrome C is concentrated much more in the left ventricle than in the auricle and the free wall of right ventricle, and also the samples from puppies contain cytochrome C about 1.4 times as much as the samples of adult dogs. This corresponds with the ratio of DNA content between the powder samples of puppies and adult dogs.

DISCUSSION

1) The probable value of nucleic acid content in the dog heart:

According to Banga *et al.* (1955)[6], the total NA-P of the rat heart is 38 mg.%. J. N. Davidson & C. Waymouth (1944)[7] reported that in the sheep heart the total NA-P content and the ratio of RNA-P to DNA-P were 29 mg.% and 3.6 for the adult, 64 mg.% and 2.4 for the embryo, respectively.

In our experiments, the probable value of the total NA-P content in the dog heart is thought as about 28 mg.% of which the RNA-P is 20 mg.%, and then the ratio of RNA-P to DNA-P becomes about 2.5 (Figs. 3 & 4).

2) DNA content and the cell size in various parts of the dog heart:

Since the DNA-P contents in the excised strips did not change during the survival *in vitro*, the DNA-P contents in various parts of the dog heart *in vivo* can be estimated by the DNA determination of the stored powders.

In view of the evidence[8][9] that nucleus of any body cell contains the same amount of DNA, the tissue, in which the DNA content is much less, is to consist of much larger cells.

Thus, it may be said that the cardiac muscle cells of adult dogs are much larger in size than those of puppies, even if the water content in the heart of puppies is about 10% much more. Also among the various parts of the puppie's heart, the free walls of the left ventricle must be constructed by larger cells than the others. During the maturation, these differences become indistinguishable, *e.g.*, it is suggested that every cardiac cell of the adult dog becomes homologous in size. The values in the bottom of Table III may present the degree of increment in cell size during the maturation.

3) The difference of nutritional condition in various parts of the dog heart:

Because of the rapid fall of RNA-P contents in the surviving strips, the values of the stored powders may be not safely appreciated as such. But, assuming that the RNA-P contents in strips of both the right and left ventricle decreased at the same rate during their survival as indicated in Figure 4, the values of stored powders may well reflect the contents of RNA-P in various parts of the dog heart.

Thus, it seems that the left ventricle, especially its endocardial part, is much more obliged than the other parts with abilities to synthesize cytoplasmic proteins and to arrange nutritional factors, because it has been demonstrated by T. Caspersson (1941)[10], H. Brachet (1942)[11], H. W. Kosterlitz (1947)[12] and others that the RNA is rich in the cells where protein synthesis is occuring actively and that their RNA amounts decrease remarkably in the unfavorable conditions of nutrition. This view is fortified with the previous data of one of the authors (1957)[1]. He showed that oxygen consumptions of the minced samples of the dog's left heart were not influenced so much by the additions of yeast extracts

and glucose and were not decreased so rapidly as the minces of the other parts.

4) The probable value of cytochrome C content in the dog heart:

Figure 2 and Table II shows that the cytochrome C contents of the strips survived in a cold buffered saline are well preserved, though in some case its initial fall is observed, and are closely comparable to even that of powder samples stored for 2 years or more after the excision. Similar facts were pointed by G. Biörk (1956)[13] and O. Rosenthal & D. L. Drabkin (1943)[14]. Considering that this enzyme is so stable, our cytochrome C determination of stored powders can present the values of various parts of the dog heart *in vivo*. Indeed, our results indicate that the value of the left heart (19 mg. %) is comparable with the data of Drabkin[15] who showed that the dog heart, probably the left heart, contained about 25 mg. % wet weight of cytochrome C.

5) Differences of cytochrome C contents in various parts of the dog heart:

Our data of the dog heart are similar with the results of G. Biörk (1956)[13] that the left ventricle of the human heart contains much more amounts of cytochrome C than the other parts of heart. In Biörk's data the ratio of the left to the right ventricular cytochrome C content (mg. % wet weight) is 47:30 (nearly equal to 5:3) and in our results it is $\frac{13.1+8.5}{2}$: 6.4 (nearly equal to 5:3) for the adult dogs and $\frac{18.8+14.6}{2}$: 7.6 (nearly equal to 5:2) for puppies, assuming that the left heart consists of equal part of the endocardial and pericardial parts.

In our experiments the auricle contained much smaller amount of cytochrome C than the free wall of the right heart, while in data of Biörk was no difference between them. According to Yagi who measured the oxygen consumption of minced samples from the various parts of the dog heart, the auricle in which the cytochrome C is the least in amount, is rather highly oxidative. Since it is usually thought that the cytochrome C is a limiting factor in the tissue oxidation, this discrepancy will be further investigated.

The values, indicated in the bottom of Table II, show the degree of decrease of cytochrome C content during the maturation. If this value exceeds the degree of increment in cell size (Table III) as in the case of the left ventricle, especially the pericardial part, it seems that the synthesis of cytochrome C is apparently discontinued. On the other hand, in the free wall of the right heart, the increase in production of cytochrome C can be expected. Thus, the reason why oxygen consumptions of the samples from the right heart is relatively stable is somewhat understood.

Summary

1) The contents of cytochrome C and nucleic acids in the fresh muscle strips and the stored powders from various parts of the dog heart were determined.

2) The left heart *in situ* contains probably 19 mg. % of cytochrome C, 8 mg. % of DNA-P and 20 mg. % of RNA-P.

3) The puppie's hearts contain 1.4 times as much DNA and cytochrome C as that of adult dogs. The RNA contents are rapidly lost after death, and also are not differentiated between both groups.

4) Among the various parts of the dog heart, both RNA and cytochrome C are concentrated predominantly in the left heart, which contains comparatively smaller amounts of DNA and then may consist of much larger cells. The auricles, which consumes oxygen most actively, contain the least amount of cytochrome C.

5) During the maturation of the dog, the right heart of which the absolute content of cytochrome C is relatively small, synthesizes this enzyme increasingly, and in contrast the left heart apparently discontinues its synthesis.

We wish to express our thanks to Prof. T. Sakurai, Research Institute for Scientific Measurements of Tohoku University, for his technical advices.

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The Tohoku Journal of Experimental Medicine, Vol. 70, No. 3, 1959

Clinical Studies on Liver Catalase Activity in Gastric Carcinoma Cases

I. Clinical Observations

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(Received for publication, May 25, 1959)

Diminished catalase activity in the liver of tumor-bearing animals was reported first by Brahn[1], and confirmed then experimentally by Greenstein *et al.*[2][3] and by many other workers. In our clinic, it has been reported that the liver catalase activity in gastric carcinoma cases is obviously lower than that in the control noncancerous cases[4].

In the mentioned report, it was also noticed that the liver catalase activities of persons with the tumor vary markedly from case to case. However, whether such a variety is due only to an individual scatter or is relating to the variety in pathological features of the tumor has not yet been clarified.

The present writer estimated the liver catalase activity of patients subjected to operation for gastric carcinoma and studied its relation with the pathological conditions of the tumor and with some laboratory findings of the patients.

Experimental

Method

1) Objects

The objects of this study were 82 cases (58 males and 24 females) operated in our surgical clinic for gastric carcinoma, their age being 21 to 76 years, 56.1 years on an average. These were itemized into the following groups: 58 cases treated with the subtotal resection of the stomach, five cardiectomy cases, eight total resection cases, four cases treated palliatively (one with gastroenterostomy and three with exploration) and seven cases with metastases to the liver.

The control group consisted of 18 cases with peptic ulcer of the stomach or duodenum. It included 14 males and four females, their age being 26 to 66 years, 44.9 years on an average.

2) Method for Estimating Liver Catalase Activity

A wedge shaped piece was cut from the ventral margin of the liver immediately after laparotomy, a parenchymatous portion of which, weighing about 0.1 g., was washed with Sörensen's phosphate buffer of pH 7.0 to remove the blood, and then weighed. It was then masticated in a mortar with a small amount of quartz sand, during which time the buffer solution was added in small quantities to make a 1:2000 suspension. After being left to stand for 12 hours in an ice chamber, the suspension was centrifuged for five minutes at 1000 G., and the supernatant thus obtained was subjected to the estimation.

Measurement of the catalase activity was performed by means of Warburg's manometry, that is, the amount of oxygen evolved in 30 minutes from a reacting system which consisted of 0.1 cc. of the mentioned enzyme solution, 0.1 cc. of 1.50 per cent hydrogen peroxide as a substrate and 2.8 cc. of the buffer was measured manometrically and the value given in μl. was used as the liver catalase activity. Temperature, 37.5°C. Agitation, by horizontal semi-circular motion of 80 strokes per minute.

Results

1) Liver Catalase Activity in Gastric Carcinoma Cases and in Controls

In Table I are summarized the results with the 82 gastric carcinoma cases and with the 18 control cases, and Fig. 1 shows the frequency distribution of the data.

The carcinoma group contained the cases with the low catalase activities in higher rate than the control group, the mean catalase activity in the former being obviously lower than that in the latter.

Liver catalase activities in seven cases accompanied with metastases to the liver were significantly lower, not only than the noncancerous cases but also than the carcinoma cases without the hepatic metastasis.

TABLE I

Liver Catalase Activity in the Total Cases Examined

Groups		Cases	Liver catalase activity		
			Minimum	Maximum	Mean*
Gastric or duodenal ulcer		18	66.8	140.9	102.1±10.9
Gastric carcinoma	without metastasis to the liver	75	35.3	112.9	74.6± 3.5
	with metastasis to the liver	7	30.4	62.0	49.4±12.5

* Arithmetical means with 95 per cent confidence ranges.

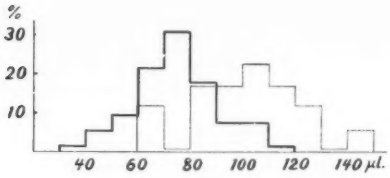

Fig. 1. Frequency distribution of liver catalase activities in 75 cases with gastric carcinoma (heavy lines) and in 18 control cases (light lines), in per cent.

2) Liver Catalase Activity of Carcinoma Cases for Various Operative Groups

Hereafter the objects of the study will be limited to 75 cases which were not accompanied with metastasis to the liver. First, the liver catalase activity in various operative groups was compared with each other.

As shown in Table II, the activities were obviously lower in four cases in which resection of the stomach could not be undertaken and only an exploration or a palliative operation was carried out, as compared with those in 71 resected cases. Among the latter, the cases that required total resection of the stomach showed remarkably lower values than the others in which a partial resection was indicated.

TABLE II

Liver Catalase Activity of Gastric Carcinoma Cases for Various Operative Groups

Operations	Cases	Liver catalase activity		
		Minimum	Maximum	Mean
Resection of the stomach	71	35.3	112.9	75.2±3.6
Subtotal resection	58	35.3	109.7	77.2±3.8
Cardiectomy	5	62.9	112.9	78.2
Total resection	8	44.0	73.2	58.6±8.8
Palliative operation or exploration	4	46.5	88.0	64.8

3) Relation of Liver Catalase Activity with Local Aspects of Cancer Lesion (See Table III).

Relation with location of the tumor. Eleven cases with carcinoma located in the cardiac region showed comparatively lower liver catalase activities than the others, whereas there was little difference between 43 pyloric and 21 corporal cancers.

Relation with the grade of peritoneal dissemination. In the table, P.C. 0 group includes the cases in which the serous membrane of the stomach was not yet involved, P.C. I those in which the carcinomatous dissemination was confined to the stomach serosa or to the omentum adjacent to

TABLE III

Liver Catalase Activity of Gastric Carcinoma Cases for Various Aspects of Lesion

Local aspects of lesion		Cases	Liver catalase activity		
			Minimum	Maximum	Mean
Location	Pylorus	43	35.3	109.7	76.2± 4.7
	Corpus	21	57.2	100.3	75.8± 5.9
	Cardia	11	44.0	112.9	65.9±12.9
Peritoneal dissemination	P.C. 0	14	48.4	109.7	85.4± 9.3
	I	30	53.8	100.3	78.4± 4.1
	II	27	35.3	112.9	67.3± 6.2
	III	4	46.5	62.4	57.8
Lymph-nodal involvement	Grade 0	11	68.1	109.7	87.2± 7.4
	I	7	67.9	100.3	82.8±10.9
	II	57	35.3	112.9	71.2± 3.9

the tumor, P.C. II those in which it extended beyond the stomach but a radical operation including resection of the omenta might be able to remove it almost completely, and P.C. III those in which it spread extensively to show a condition named carcinomatous peritonitis.[5]

The relation between the liver catalase activity and the peritoneal disseminations of gastric carcinoma was striking. The catalase activities were highest in P.C. 0 group, lowering in turn in P.C. I, II and III groups.

Relation with the grade of lymph-nodal involvement. The catalase activity correlated also with the grade of lymph-nodal involvement. Thus, the activities in cases without lymphatic metastasis (grade 0) were apparently higher than those with the metastases to the stomach nodes (grade I) or up to the retroperitoneal nodes (grade II).

4) Relation of Liver Catalase Activity with Types of Tumor

In Table IV are shown the liver catalase activities for various types of the tumor in 71 cases in which resection of the stomach was performed.

Relation with the size of the tumor. Size of the tumor was measured in the resected fresh stomach and designated, for convenience, as follows according to the product of the longitudinal diameter by the transverse one: the "small tumor", under 20 cm.^{2}; the "medium tumor", 20 cm.^{2} or more but less than 40 cm.^{2}; the "large tumor", 40 cm.^{2} or more.

Then, there were some relations between the catalase activity and the tumor size, cases with larger tumor tending to show somewhat lower

TABLE IV

Liver Catalase Activity in Gastric Carcinoma Cases in which Resection of Stomach was Performed

Types of tumor		Cases	Liver catalase activity		
			Minimum	Maximum	Mean
Size	Small	20	59.4	103.4	83.3±6.2
	Medium	19	48.4	109.7	75.7±7.7
	Large	32	35.3	112.9	70.0±5.2
Gross appearance	Type I	4	76.5	91.4	81.9
	II	43	35.3	112.9	73.7±5.1
	III	19	59.4	104.7	79.3±5.8
	IV	5	48.9	94.6	66.5
Histological feature	Adenomatosum	30	35.3	109.7	76.2±6.0
	Solidum	40	44.0	112.9	77.1±4.8

activities than those with smaller tumors.

Relation with the gross appearance. When the tumors were grouped according to their gross appearance (Borrmann), the number of cases was too small for statistical comparison in some groups. But, no significant difference was found in catalase activity between 47 cases with comparatively circumscribed tumors (types I or II, in which the mean liver catalase activity was 74.4±4.7) and 24 cases with infiltratinglty growing tumors (types III or IV, 76.6±6.1), nor between 62 cases with ulcerated tumors (types II or III, 75.4±3.9) and nine cases with non-ulcerated tumors (types I or IV, 73.7±12.5).

Relation with the histological feature. Thirty cases with carcinoma adenomatosum (Petersen) presented as much catalase activities as 40 cases with carcinoma solidum, the activity in the remaining one (the mixed type) being 73.6.

5) Liver Catalase Activity and Various Laboratory Findings in Gastric Carcinoma Cases

Erythrocytes studies were carried out in all of the 75 gastric carcinoma cases and the electrophoretic analyses of serum protein (Tiselius) in 42 cases out of them. And, according to every one of the data obtained, the cases were grouped into four classes, I to IV, each of them including one-fourth of the total cases. Three boundaries between the adjoining two classes (viz. quartiles) are shown in Table V.

Besides, the serum colloidal reactions and the bromsulphalein test were carried out as the function tests of the liver, in 65 and in 41 cases respectively.

TABLE V

Laboratory Findings of Gastric Carcinoma Cases Examined

Tests	Cases	Mean	Quartiles		
Erythrocyte count (millions per μl.)	75	407±15	365	416	448
Hematocrit (per cent)	75	40±2	36	41	45
Hemoglobin concentration (g/dl.)	75	12.3±0.2	10.7	12.8	14.3
Mean corpuscular volume (cubic microns)	75	98.1±2.9	90.8	97.4	106.1
Mean corpuscular hemoglobin ($\gamma\gamma$.)	75	30.4±1.0	27.7	31.3	32.6
Total concentration of serum protein (g/dl.)	42	6.8±0.2	6.3	6.8	7.2
Albumin-globulin ratio	42	1.01±0.03	0.94	1.01	1.09

Erythrocyte. See Table VI. Liver catalase activities were comparatively low in cases in which the number of red blood corpuscles was small. An intimate correlation of the two values is given also in Fig. 2.

The relation between the catalase activity and the hematocrit was similar to the case of the erythrocyte count. Though some correlation was assumed between the catalase activity and the mean corpuscular volume, a mathematical scrutiny revealed it to be insignificant.

The liver catalase activity also correlated markedly with the hemoglobin concentration, the lower catalase activity being found in cases with the lower hemoglobin level (Fig. 3). In addition, cases with comparatively low mean corpuscular hemoglobin (classes I or II) were generally accompanied with the lower catalase activity as compared with those with the moderate or rather high MCH (classes III or IV).

Serum protein. See Table VII and Fig. 4. Cases accompanied with hypoproteinemia (classes I or II) showed apparently lower catalase activities than those in which the total concentration of serum protein was normal or higher (classes III or IV). Cases with low albumin-globulin ratio (classes I, II or III) presented markedly low activities, while those with the nearly normal ratio comparatively high activities.

Liver function. See Table VIII. Colloidal reactions performed were the thymol turbidity test, zinc sulfate test and Sawada and So's sublimate reaction[6], positive reaction of them being determined with the values over 2.0 units, over 12.0 units and under 0.65 cc., respectively. Among them the thymol turbidity test and the zinc sulfate test gave only a few positive cases, the relation of them to the liver catalase activity being not clarified. In sublimate reaction, which was positive in about a half of the total, positive cases accounted for nine out of 10 cases in which the catalase activities were less than 60, and 15 out of 24 cases with the activities under 70.

There was no marked difference in the liver catalase activity between

TABLE VI

Liver Catalase Activity for Various Hematological Conditions of Gastric Carcinoma Cases

Hematology	Classes	Cases	Liver catalase activity		
			Minimum	Maximum	Mean
Erythrocyte count	I	19	35.3	112.9	68.0±7.2
	II	19	46.5	93.6	72.6±7.3
	III	19	53.8	104.7	75.5±6.5
	IV	18	44.0	109.7	82.8±7.9
Hematocrit	I	19	35.3	112.9	67.0±8.5
	II	19	48.4	92.7	72.0±5.8
	III	19	44.0	104.7	76.5±6.0
	IV	18	57.2	109.7	83.4±6.9
Mean corpuscular volume	I	19	44.0	103.4	71.8±8.3
	II	19	35.3	104.7	73.0±8.8
	III	19	48.9	100.3	75.8±6.9
	IV	18	48.4	112.9	77.9±7.9
Hemoglobin concentration	I	19	35.3	112.9	68.8±8.1
	II	19	44.0	92.7	70.7±6.9
	III	19	48.9	94.2	72.8±5.3
	IV	18	58.9	109.7	87.2±6.7
Mean corpuscular hemoglobin	I	19	35.3	103.4	70.0±8.7
	II	19	46.5	94.6	70.5±6.2
	III	19	48.9	112.9	79.7±7.4
	IV	18	57.2	100.3	78.6±6.9

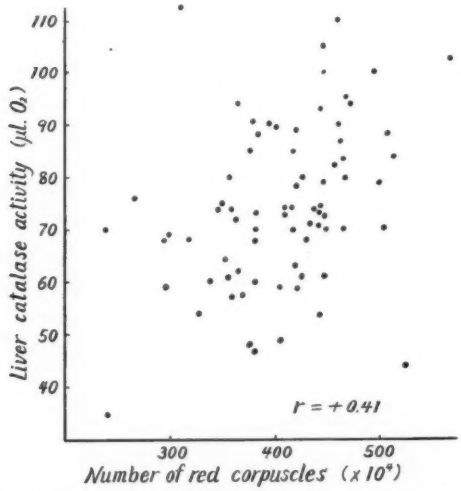

Fig. 2. Correlation between the liver catalase activity and the erythrocyte count, in 75 gastric carcinoma cases.

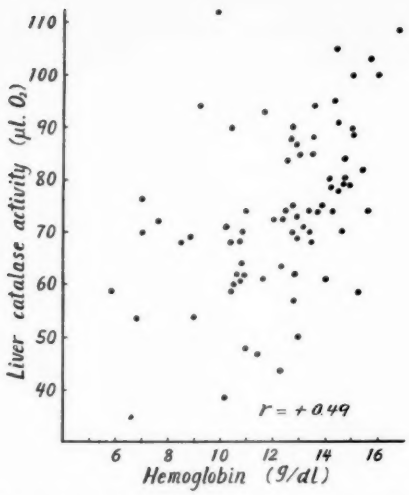

Fig. 3. Correlation between the liver catalase activity and the hemoglobin concentration, in 75 gastric carcinoma cases.

TABLE VII

Liver Catalase Activity of Gastric Carcinoma Cases According to Pictures of Serum Protein

Serum protein	Classes	Cases	Liver catalase activity		
			Minimum	Maximum	Mean
Total concentration	I	11	48.9	89.8	70.2±6.9
	II	10	35.3	103.4	68.6±12.7
	III	10	57.2	91.4	74.5±7.7
	IV	11	53.8	112.9	85.6±13.3
Albumin-globulin ratio	I	11	53.8	103.4	73.3±9.9
	II	10	35.3	112.9	73.2±12.5
	III	10	44.0	91.4	70.8±11.7
	IV	11	59.4	104.7	80.8±10.8

positive cases of bromsulphalein test (being judged to be positive when the stagnation level was 7.0 per cent or more after 30 minutes) and the negative ones.

DISCUSSION

As the lowering of the liver catalase activity is generally observed

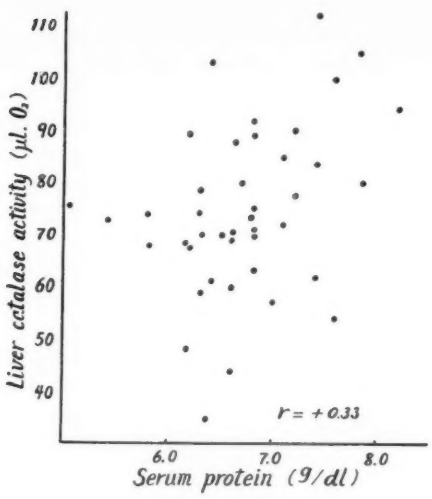

Fig. 4. Correlation between the liver catalase activity and the total concentration of serum protein, in 42 gastric carcinoma cases.

TABLE VIII

Liver Catalase Activity and Functions of Liver in Gastric Carcinoma Cases

Liver function* ↓ / Catalase activity→		~50	50~60	60~70	70~80	80~90	90~100	100~	Total	Mean catalase activity
TTT	+	1		2	2	1			6	
	−	3	6	12	18	10	5	5	59	
ZST	+			1		2			3	
	−	4	6	13	20	9	5	5	62	
SR	+	4	5	6	11	4	2		32	69.5±5.5
	−		1	8	9	7	4	4	33	79.7±5.0
BSP	+		2	1	3	4	1		11	76.5±7.9
	−	3	3	7	8	4	2	3	30	73.1±6.6

* TTT, thymol turbidity test; ZST, zinc sulfate test; SR, sublimate reaction; BSP, bromsulphalein test.

in various tumors including those far from the liver or those implanted experimentally, and is restored by extirpation of the tumor[3], it is thought to be one of the far-reaching effects of the tumor. In this work, the relation of the lowered catalase activity of the liver to the local progress of the tumor as well as to such general conditions in cancer as anemia, hypo-

proteinemia and the like was studied clinically, in gastric carcinoma cases treated in our clinic.

Local aspects of gastric carcinoma are various and can be classified from various points of view. What is the nature of the tumor, on which the lowering of the liver catalase activity is closely dependent? To slove the question, it may be rational to compare the liver catalase activities of the various groups of cancer cases with each other.

First, it was revealed that the cases in which resection of the stomach could not be undertaken and only exploration or palliative anastomosis was performed showed far less catalase activities than the resected cases, and among the latter, cases in which total gastrectomy was required than those treated with partial resection of the stomach.

In the surgery of cancer, the mode of operation is selected, needless to say, mainly from the viewpoint of resectability and radicality and also under consideration on general conditions. Therefore, the operative procedure indicated to a patient is one of the most simple indicators showing the grade of cancer progress in him. In this sense, the above-mentioned result may be said to suggest the close relation between the liver catalase activity and the grade of cancer progress.

The main local factors of gastric carcinoma which decide the resectability and radicality are the location of the tumor and the extent of the cancer spread, either peritoneal or lymphatic, the size as well as morphological feature of the tumor being of little value in this point. The present work showed that the liver catalase activity is lower in cases with cardiac cancer than in the others, and is lower also in those with the more extensive peritoneal dissemination or the more advanced lymphatic metastases.

For interpreting the results, however, some discussion is needed, that is, the interdependence of the factors must be taken into consideration. In fact, surgical cases of cardiac cancer are apt to show comparatively advanced spread, presumably because of the difficulty in early diagnosis due to anatomical peculiarities, and this was true in cases of this work (Table IX). Therefore it is thought that comparatively low catalase activity in such cases is due not to the cardiac situation of the tumor itself but to the comparatively high content of the progressed cases.

Peritoneal dissemination and lymph-nodal involvement are both the main routes for generalization of gastric cancer and are responsible for unfavorable prognoses in progressed cancer cases.[5] Which of the two is likely to be more essential seems not to be of practical importance. It may be natural to conclude here that the lowering of the liver catalase activity is closely dependent upon the extent of cancer spread, that is, to the grade of peritoneal dissemination as well as lymphatic metastasis.

TABLE IX

Rate of Progressed Cancer Cases According to Location and Size of Tumor

Location or size	Cases	Peritoneal dissemination of P.C. II & III	Lymph-nodal involvement of grade II
Pylorus	43	15 (34.9%)	30 (69.8%)
Corpus	21	8 (38.1%)	17 (81.0%)
Cardia	11	8 (72.7%)	10 (90.9%)
Small	20	3 (15.0%)	11 (55.0%)
Medium	19	6 (31.6%)	13 (68.4%)
Large	32	18 (56.3%)	29 (90.6%)

Liver catalase activities in gastric carcinoma cases, on the other hand, had no significant relation with the macroscopic or microscopic features of the tumor. This suggests that the difference in morphological types of the tumor is not an essential factor resulting in variety of clinical manifestations due to the tumor, that is, the predominating factor on this point may be not the morphological nature of the tumor itself but the invading potential of the tumor which is realized as the peritoneal or lymphatic spreads.

A little tendency was observed that the less catalase activities were found in cases with larger tumors. This seems to be concordant with the experimental results of Adams[7] and Weil-Malherbe *et al.*[8] However, as the cancer spreads were generally more advanced in cases with larger tumors (Table IX), some studies should be required to decide whether the low catalase activities in cases with large tumors are related to dimensions of the tumor per se. On this matter another report will be published.

Gastic carcinoma patients, especially in the middle or the later stage of progress, show various general symptoms, some of which are common to the malignant tumors of various organs and are thought to be early manifestations of cancer cachexia. If the lowering of the liver catalase activity is one of the patho-physiological effects of the tumor, is there any relation between this phenomenon and the other general symptoms of cancer?

In answer to this problem, the present work showed that the liver catalase activities of gastric carcinoma cases were particularly low in those with anemia, especially in hypochromic cases, and in addition, in cases with hypoproteinemia or with hypalbuminemia.

As the prosthetic group of a catalase molecule is protohemin, one of the iron-porphyrin bodies, there are theories that the lowering of catalase activity in cancer is due to the disturbed iron metabolism and is correlative with the decrease in hemoglobin, yet opinions diverge on this mat-

ter.[9][10][11] A clinically proved parallelism of the two phenomena, the lowering of the liver catalase activity and the anemia in tumor-bearing persons, may provide a suggestion to this problem, even though it can not indicate directly the physiological correlation of them.

As to the relation of the lowering of the catalase activity to the disturbed protein metabolism in tumor-bearing animals, Miller[12] believed that the synthesis of catalase is depressed through deficiency of protein component of this enzyme, and, on the contrary, Weil-Malherbe *et al.*[8] failed to detect any relation between those two phenomena, suggesting that each of them is one of the various disturbances elicited from the existence of the tumor.

Besides, it may be also possible that the depressed catalase activity affects directly the synthesis of protein in the liver. In fact, the liver is undoubtedly the main source of serum protein and probably an only source of serum albumin[13], and on the other hand, catalase has been proved to be an enzyme of biological significance, notwithstanding an old opinion that it only decomposes hydrogen peroxide to protect the tissue from toxic effects of this substance[14]. Which one of these mechanisms concerns the human carcinoma must be confirmed by future research.

The next subject to be discussed is whether the lowered catalase activity has any influence upon the figures in clinical function tests of the liver. As catalase is a metabolic enzyme contained abundantly in the liver, lowering of its activity may possibly result in a fall of the function of this organ. Though there was a test (sublimate reaction) suggesting this relation, some tests were too low in their positive rate in cancer cases including those with markedly depressed catalase activity, or were wholly irrespective of the latter. This emphasizes that, in cancer cases, the function tests for the liver should be carried out from various angles and that the negative results of a few tests can not always indicate the intact liver function.

In conclusion some discussions will be made on the possible value of estimating catalase activity for early diagnosis of cancer.

Recently, measurement of the catalase activity has been attempted on the biopsied liver tissue in some cases with hepatic disorders.[15] Apart from such questions as invasive influences exerted upon patients by needle-biopsy, poor homogeniety of the material and technical difficulty due to small material, is this procedure able to contribute to earlier diagnosis of cancer?

Though the difference in the liver catalase activity was obvious between the carcinoma group and the control one, the distribution ranges of the two groups were separated from each other not sharply but only transitionally, there being an area common to both groups (See Fig. 1).

And, that early cancer cases should be included in such an area is easily presumable from the results of this work.

Actually, catalase activities in 16 cases in which both the peritoneal and the lymphatic spreads were limited in the stomach region were 67.9 to 109.7, all of them being included in the distribution range of non-cancerous cases, 66.8 to 140.9. Those in which the liver catalase activity was far lower than the controls were progressed cases, diagnosis of which was easy by the usual clinical examinations only.

Such a difficulty in early diagnosis is anticipated also even when we attempt the screening of cancer cases by means of measuring the liver catalase activity. The rejection limits of catalase activities in gastric carcinoma calculated from the 75 examined cases were 74.6 ± 31.2 at five per cent probability of the possible error. When we take the superior limit 105.8 of this range as the maximal value of positive results of the screening, the majority of gastric carcinoma cases will, of course, be positive, but on the other hand, about half of the cases without cancer will be positive too.

Detection of lowered catalase activity is thus thought to be of little value as a method for earlier diagnosis of cancer.

Summary

Catalase activity of the liver was measured in 82 cases with gastric carcinoma as well as in 18 cases with peptic ulcer, resulting in the following conclusions.

1. Liver catalase activity is generally lower in gastric carcinoma cases than in the controls.

2. In gastric carcinoma cases, the lower catalase activities are observed in cases with the more progressed lesions, that is, in cases with the more extended disseminations and in those with the more widely involved lymph nodes, while the activities have little relation to the morphological features of the tumor themselves.

3. In gastric carcinoma cases, the liver catalase activities are comparatively low in those with severe anemia and in those with hypoproteinemia.

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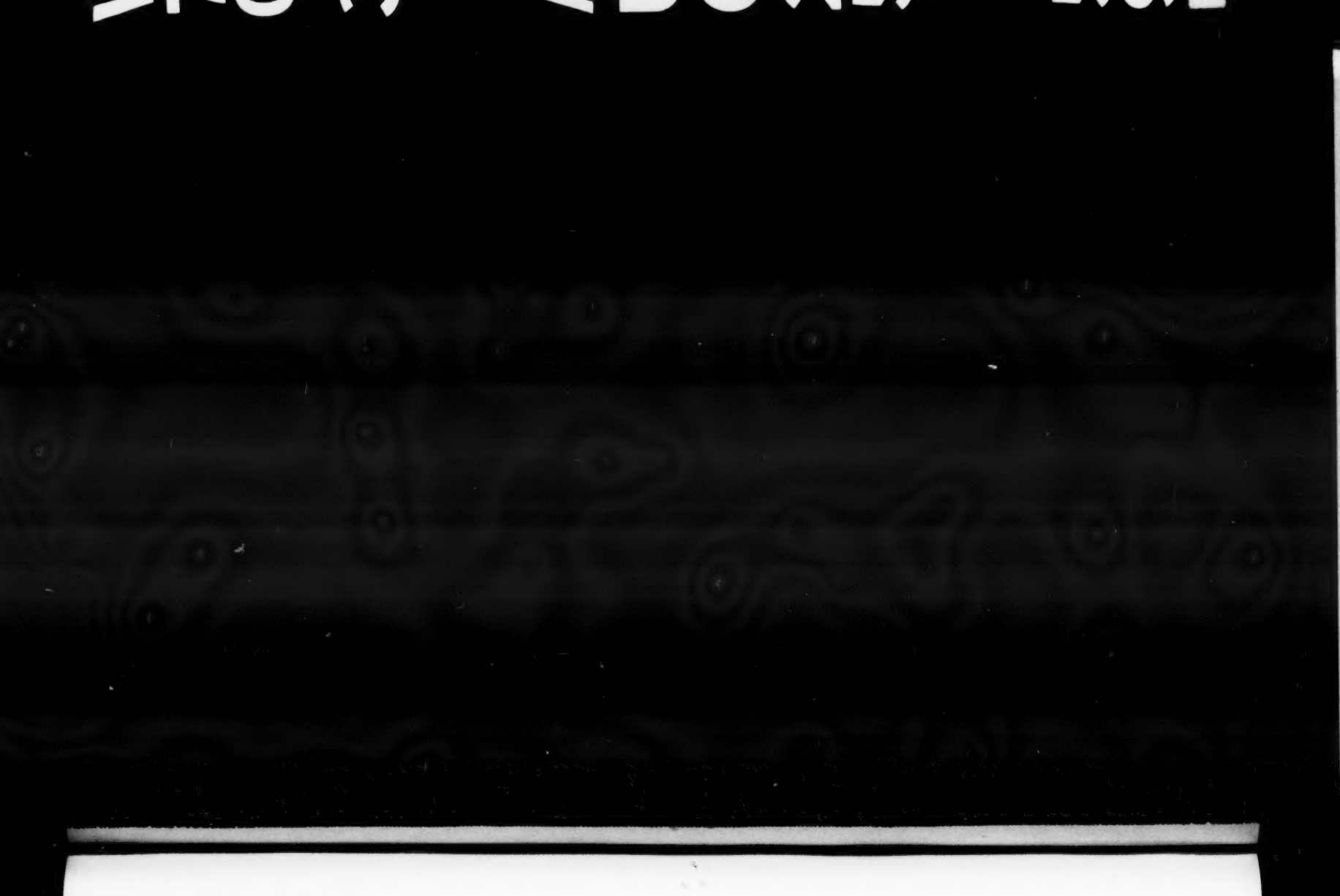
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